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# Microbial Diversity in Ultra-High-Pressure Rocks and Fluids from the Chinese Continental Scientific Drilling Project in China

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**Microbial communities in ultra-high-pressure (UHP) rocks and drilling fluids from the Chinese Continental Scientific Drilling Project were characterized. The rocks had a porosity of 1 to 3.5% and a permeability of ~0.5 mDarcy. Abundant fluid and gas inclusions were present in the minerals. The rocks contained significant amounts of $Fe_2O_3$, FeO, $P_2O_5$, and nitrate (3 to 16 ppm). Acridine orange direct counting and phospholipid fatty acid analysis indicated that the total counts in the rocks and the fluids were $5.2 \times 10^3$ to $2.4 \times 10^4$ cells/g and $3.5 \times 10^8$ to $4.2 \times 10^9$ cells/g, respectively. Enrichment assays resulted in successful growth of thermophilic and alkaliphilic bacteria from the fluids, and some of these bacteria reduced Fe(III) to magnetite. 16S rRNA gene analyses indicated that the rocks were dominated by sequences similar to sequences of *Proteobacteria* and that most organisms were related to nitrate reducers from a saline, alkaline, cold habitat; however, some phylotypes were either members of a novel lineage or closely related to uncultured clones. The bacterial communities in the fluids were more diverse and included *Proteobacteria*, *Bacteroidetes*, gram-positive bacteria, *Planctomycetes*, and *Candidatus* taxa. The archaeal diversity was lower, and most sequences were not related to any known cultivated species. Some archaeal sequences were 90 to 95% similar to sequences recovered from ocean sediments or other subsurface environments. Some archaeal sequences from the drilling fluids were >93% similar to sequences of *Sulfolobus solfataricus*, and the thermophilic nature was consistent with the in situ temperature. We inferred that the microbes in the UHP rocks reside in fluid and gas inclusions, whereas those in the drilling fluids may be derived from subsurface fluids.**

Advances in our understanding of the origins, diversity, distributions, and functions of microorganisms in deep, often extreme, subsurface environments are rapidly expanding our knowledge of biogeochemical processes on Earth and beyond. The discovery of novel microorganisms in deep accessible subsurface habitats provides opportunities for discovering new pharmaceuticals, studying biosynthetic processes, remediating contaminated environments, and enhancing energy production. The major obstacles to understanding the subsurface biosphere have been our limited abilities to access the deep subsurface environment, to acquire uncontaminated samples, and to place our knowledge of microorganisms (functional genes and proteins) into an environmental context. Past and current opportunities to address biogeochemical processes have largely been limited to the shallow crust and geographically sparse locations (7, 19, 30, 32). More recently, Onstott et al. (27) studied microbial diversity, abundance, and functions in the metamorphic quartzite and Carbon Leader from deep mines in South Africa. Despite the great caution taken, the authors demonstrated that the samples were still contaminated, but they were able to show that indigenous microorganisms were also present at concentrations of $<10^2$ cells/g and $\sim10^3$ cells/g for the Carbon Leader and quartzite, respectively.

A unique opportunity became available to us from the Chinese Continental Scientific Drilling (CCSD) Project, the world's deepest ongoing drilling in China (http://www.icdp-online.de/sites/donghai/news/news.html). Employing the most recent drilling technologies, the CCSD Project, sponsored by the International Continental Drilling Program and the Chinese government, is a project to drill a 5,000-m-deep borehole in the eastern part of the Dabie-Sulu ultra-high-pressure (UHP) metamorphic orogenic belt that is located at the center of the convergent plate boundary between the Sino-Korean Plate and the Yangtze Plate. The CCSD Project provides a unique opportunity to study deep subsurface microbiology by offering (i) a 5,000-m continuous drill core across a wide range of environmental gradients and (ii) a truly multidisciplinary international research team that ensures measurements of geological, geochemical, and hydrological parameters that are essential to interpretations of microbial studies.

The goal of this study was to systematically investigate the microbial diversity and abundance in UHP rocks from depths of 529 to 2,026 m by culture-dependent and -independent approaches. Our results suggest that there is a unique microbial community in the deep rocks and geological fluids, and the measured and inferred functions of cloned sequences are consistent with the geochemical environments. Our study extends the current investigations of the subsurface microbiology into high-pressure metamorphic rocks in a unique tectonic setting.

## MATERIALS AND METHODS

**Site description and geology.** The CCSD Project drilling site is located in Donghai County, Lianyungang City, Jiangsu Province, in the eastern part of the Dabie-Sulu ultra-high-pressure metamorphic belt (Fig. 1). The Dabie-Sulu ultra-

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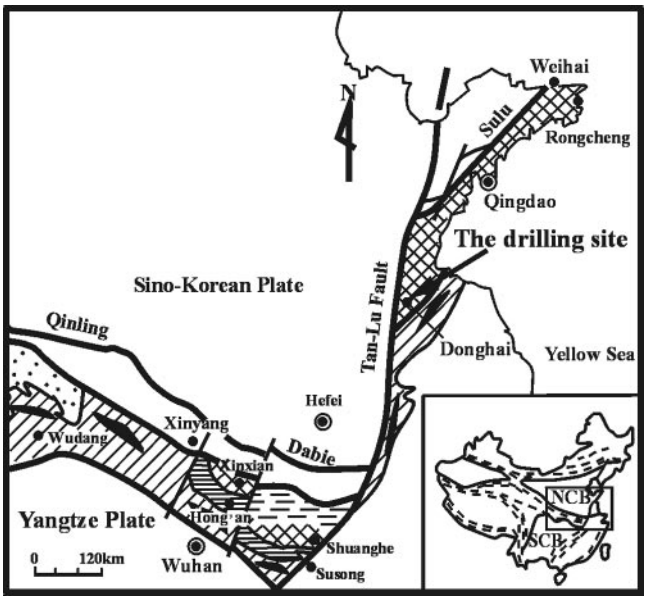



FIG. 1. Map showing the general geology in the Dabie-Sulu orogen of central-eastern China. NCB and SCB in the inset indicate the North China Block (part of the Sino-Korean plate) and the South China Block, respectively. The drilling site is the circle labeled Donghai. After Zheng et al. (44).

high-pressure metamorphic belt was formed by a collision between the Sino-Korean Plate and the Yangtze Plate about 240 million years ago. The occurrence of diamond and coesite in the rocks reveals that temperature-pressure conditions were 700 to 850°C and 2.8 GPa for the UHP metamorphism (44). The current geothermal gradient at the site is approximately 25°C/km.

The UHP rocks were subducted to a depth of at least 100 km and experienced UHP metamorphism before they were rapidly brought to the surface about 220 million years ago. By drilling to a depth of 5 km, it is possible to study rocks that used to be at a depth of 100 km. The products of plate tectonics of this region (i.e., UHP rocks and minerals), along with abundant fluids, radioactivity (U and Th), and gases ($H_2$, $CO_2$, CO, and $CH_4$), have provided a unique environment for subsurface microbes. The UHP rocks are typically separated by a series of structurally weak shear zones and faults. These shear zones and faults are potential storage spaces for large pockets of fluids and gases, and they may serve as potential microbial habitats. Many rocks also contain fluid and gas inclusions, and they are also potential habitats for microbes, especially when they contain carbon sources and essential nutrients, such as P and N.

**Sample collection and preparation.** Using a diamond wireline coring system, samples of rock cores and drilling fluids (from the same depth) were collected every 50 m using sterile tools, purged in an anaerobic glove box, and immediately preserved in a −80°C freezer at the drilling site. Exceptions to the sampling interval were made when geochemical anomalies were encountered, such as structure shear zones and certain depths with large amounts of fluid and gas. In such cases, the sampling intervals were closer together. The extent of surface contamination was assessed using strict quality control protocols (see below). Rock cores were 156 mm in diameter and variable in length. Twelve pairs of frozen samples of rock cores and drilling fluids covering the depth from 529 to 2,026 m below the surface were shipped to the United States in dry ice, and five of the samples (CCSD_RK529, CCSD_RK730, CCSD_RK1080, CCSD_RK1930, and CCSD_RK2026 from 529, 730, 1,080, 1,930, and 2,026 m below the ground surface, respectively) were analyzed to determine their geochemistry and microbiology. Given the measured geothermal gradient, the in situ temperatures for these rocks were 38, 43, 52, 73, and 95°C, respectively. As an example, the designation of the CCSD_RK529 sample indicates the following: CCSD, Chinese continental scientific drilling; RK, rock; 529, depth (in meters) at which the rock was located. The designations of the drilling fluids are the same as those for the rocks, except that RK is replaced by DF (drilling fluid).

**Quality control.** To estimate the extent of the drilling fluid intrusion into the rock cores, multiple approaches were used to address contamination issues, as follows: (i) chemical tracers, in which the drilling fluid contained abundant chloride and sulfate tracers that were used as tracers to detect possible contamination of the rock cores by the drilling fluid; (ii) microbiological tracers, in which selected rock cores were immersed in suspensions of positive control microorganisms ($10^8$ cells/ml of *Escherichia coli* and *Shewanella putrefaciens* CN32) for 1 week at 25°C and the DNA was extracted and amplified; (iii) isotope tracers, in which the total carbon (TC) and total organic carbon (TOC) concentrations and the respective $^{13}C$ values in the rock and drilling fluid samples were compared; (iv) phospholipid fatty acid (PLFA) analyses, in which one eclogite sample (S17 from 1,179 m) and several drilling fluid samples were used to compare PLFA profiles; (v) contamination possibly introduced in the laboratory procedures was tested using 70% ethanol- and oven-sterilized (500°C overnight) rock cores; and (vi) surface soil samples were collected as possible contaminant sources, and their microbial communities were determined along with those of the rock samples. Similarities or differences between the contamination source and the rock cores indicated the extent of contamination.

**Drill site petrophysical and geochemical analyses.** Rock porosity, permeability, electrical resistivity, and magnetic susceptibility were measured using various instruments in a petrophysics laboratory at the drilling site. A small subcore with two polished ends was used for determination of porosity and permeability with a gas ($N_2$ or Ar) source. Gas concentrations ($CO_2$, $CH_4$, $H_2$, and He) in subsurface fluids from shear zones or faults at various depths were determined by real-time gas chromatography. Because circulating drilling fluid was used for drilling, any gases or fluids from geological shear zones or faults were mixed with the drilling fluid; their concentrations were therefore determined by subtraction of the background noise from the measured signal. Levels significantly greater than the background level were considered to reflect gases from geological environments, most likely from structurally weak shear zones and faults in the borehole.

**Laboratory geochemical analyses.** To identify the mineralogy of the rock samples, powder X-ray diffraction patterns were obtained with a Scintag X1 powder diffractometer system using CuKα radiation with a variable divergent slit and a solid-state detector. The routine power was 1,400 W (40 kV, 35 mA). Low-background quartz X-ray diffraction slides (Gem Dugout, Inc., Pittsburgh, Pa.) were used. For analysis, powder samples were tightly packed into the well of the slides. Minerals were identified using the search-match software. Well-polished thin sections were prepared for the rock samples to perform optical microscopy observations in order to determine textural relationships of various minerals and to observe fractures and fluid inclusions. Electron microprobe analyses were performed on individual minerals of two samples (CCSD_RK529 and CCSD_RK730) for quantitative determination of the chemical composition, especially the $P_2O_5$ content and Fe(III)/Fe(II) ratio. Chemical compositions of minerals were determined from thin sections of the two rock samples with a Cameca SX50 electron microprobe (wavelength dispersive system) at the University of South Carolina. The standards used included omphacite as a standard for Na, garnet as a standard for Mg, Al, Si, Ca, and Fe, apatite as a standard for P, ilmenite as a standard for Ti, chromite as a standard for Cr, $MnO_2$ as a standard for Mn, and microcline as a standard for K. Minerals were analyzed with a focused beam in spot mode at an accelerating voltage of 15 kV and a beam current of 10 nA. For P, the peak and background counting times were set at 60 and 30 s, respectively. For other elements, the peak and background counting times were set at 20 and 10 s or at 50 and 25 s. Back-scattered electron images were taken to show fractures and various mineral phases. All five samples were analyzed for whole-rock chemistry by the wet chemistry method.

Selected samples of the rocks and the drilling fluids were analyzed for TC and TOC. The total carbon and $^{13}C$ analyses were performed with powdered samples by the combustion method with an Isochrom continuous-flow stable isotope mass spectrometer (Micromass) coupled to a Carlo Erba elemental analyzer (CHNS-O EA1108) at the University of Waterloo. The amount of carbon needed for accurate analysis was in the range from 0.03 to 0.2 mg, which was equivalent of approximately 100 mg of rock powder. For the total organic carbon analyses, powdered samples were treated with 5% HCl at 50°C overnight to remove inorganic carbonates, and this was followed by the procedure described above. The precision of $^{13}C$ analyses was better than ±0.3‰. The pore water chemistry of the rock samples, most likely from fluid inclusions, was determined by extraction with distilled water of 1 g of rock powder overnight. The rock leachate and supernatant of the drilling fluid samples were analyzed for F, Cl, $NO_3^-$, and $SO_4^{2-}$ by high-performance liquid chromatography. The pH of the drilling fluids was also measured with a pH probe.

**Direct microscopic counts.** The rock cores were split using a sterile hydraulic splitter, and the external surfaces of the cores were removed. Internal fresh nuggets (200 to 500 g) were recovered (representing ~20% mass recovery), and they were manually ground in a sterile mortar in the presence of liquid $N_2$. The resulting rock powder was divided into two aliquots; one aliquot was used for

direct counting, and the other was used for DNA extraction. Microbial cells were first detached from the rock powder by strong agitation in a 0.6% NaCl solution for 10 min (3), and this was followed by acridine orange staining and counting with an epifluorescence microscope.

**PLFA analyses.** PLFA were analyzed for one eclogite sample from the intermediate depth (CCSD_RK1179 from 1,179 m) and two drilling fluid samples (CCSD_DF730 from 730 m and CCSD_DF1080 from 1,080 m). All the samples were shipped frozen to Microbial Insights, Inc. (Rockford, TN) and the University of Georgia. Prior to analysis, the surfaces of the core were removed. The total lipid was extracted (42), and this was followed by separation of the polar lipids by column chromatography (13). The polar lipid fatty acids were derivatized to fatty acid methyl esters, which were quantified by gas chromatography (33). Fatty acid structures were verified by chromatography-mass spectrometry and equivalent chain length analysis.

**Enrichment.** Based on the similarity of cloned sequences (see below) to cultured bacteria, various media were prepared to determine whether microorganisms could be cultured from the rock and drilling fluid samples. Enrichments were set up for one representative rock (eclogite from 2,000 m) in minimal medium M1 (18) and FWA-Fe(III) medium (23) under strictly anaerobic conditions at incubation temperatures of 37°C, 60°C, 80°C, and 100°C. Fe(III) in hydrous ferric oxide was provided as the sole electron acceptor, and acetate or lactate was provided as the sole electron donor. Enrichments were also prepared for one drilling fluid sample from a depth of 2,030 m (CCSD_DF2030) using three media for anaerobic, thermophilic, and alkaliphilic bacteria from various subsurface environments. Two incubation temperatures were used, 37°C and 68°C. The first medium was an enrichment medium (designated AG medium) for *Anaerobranca gottschalkii*, a thermoalkaliphilic bacterium that grows anaerobically at a high pH (pH 9.5) and temperature (37°C) (31). The carbon source was starch or xylan. The second medium was an enrichment medium (designated CL medium) for *Caldicellulosiruptor lactoaceticus*, an extremely thermophilic, cellulolytic, anaerobic bacterium from an alkaline hot spring in Hverageroi, Iceland (26). The carbon source was microcrystalline cellulose. The third medium was an enrichment medium (designated TE medium) for *Thermoanaerobacter ethanolicus*, a thermophilic metal-reducing bacterium from the deep subsurface of the Piceance Basin, Colorado (34). The carbon source was acetate or pyruvate, and the electron acceptor was Fe(III) in oxyhydroxide. All incubations were carried out in the dark without shaking. Growth was monitored by acridine orange direct counting and by visual inspection of color change and precipitation. When growth was evident, transfer of enrichment tubes was carried out. In Fe(III) reducing medium [M1, FWA-Fe(III), or TE medium], production of biogenic Fe(II) was measured by the Ferrozine assay (39). Isolation was performed inside a glove box by pouring anaerobic agar onto plates. For isolation of thermophilic bacteria, a high-melting-point agar, GELRITE gellan gum (Sigma), was used with the roll tube method (16).

**DNA isolation, amplification, cloning, and sequence analyses.** Genomic DNA was extracted from either isolates or the samples of preserved rocks, the drilling fluids, and the surface soil sample. DNA extraction from isolates was accomplished with an UltraClean soil DNA isolation kit (Mo Bio Laboratory Inc., Solana Beach, CA). DNA extraction from the rocks, the drilling fluids, and the surface soil was carried out with a combination of physical bead beating, chemical extraction, and biological lysis. All stock and working solutions and water used for reagent preparation were filtered through a 0.2-μm filter and then autoclaved. All glassware and utensils used for nucleic acid extraction were baked at 500°C overnight. Plasticware was autoclaved. The rock powder was suspended in 82 ml extraction buffer (200 mM NaCl, 200 mM Tris, 2 mM sodium citrate, 10 mM $CaCl_2$, 50 mM EDTA; titrated to pH 8.0 with HCl) and incubated for 30 min at 37°C in a solution containing 1 ml lysozyme (100 mg/ml), 1 ml poly(A) (10 mg/ml), and 3 ml 10% pyrophosphate. The extraction solution was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol, washed with 75% ethanol, and resuspended in sterile distilled water. The resuspended solution was purified with phenol-chloroform-isoamyl alcohol (24:24:1) and precipitated with ethanol again. The crude nucleic acid extracted was purified with a QIAGEN RNA/DNA Midi kit (QIAGEN Inc., Chatsworth, CA).

Purified DNA was used as a template for amplification of the 16S rRNA gene by PCR according to the procedure described for a Failsafe kit (Epicenter Communications Inc., Sausalito, CA). Because the rock cores contained very small amounts of DNA, it was not always reliable to determine the DNA concentration by spectrophotometry. Our best estimate of the amount of DNA template was on the order of 0.5 to 1 ng/μl, and in a typical reaction, 1 μl template was added to the reaction mixture. The PCR conditions for bacteria were 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 200 μM, each primer at a concentration of 0.2 μM, and 1.25 U FailSafe PCR enzyme mixture in each 50-μl reaction mixture. The bacterial primer sequences were as follows: Bac27F, 5′-AGAGTTTGATC MTGGCTCAG; and Univ1492R, 5′-CGGTTACCTTGTTACGACTT. The following standard conditions were used for amplification of the bacterial 16S rRNA gene: 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min. Several tubes were combined to obtain enough PCR products. The archaeal primer sequences were as follows: Arch21F, 5′-T TCCGGTTGATCCYGCCGGA; and 958R, 5′-YCCGGCGTTGAMTCCAATT. The following standard conditions were used for amplification of the archaeal 16S rRNA gene: 45 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min. The amplified 16S rRNA gene fragments were ligated into the pGEM-T vector (Promega Inc., Madison, WI), and the resulting ligation products were used for transformation into *E. coli* DH5α competent cells. 16S rRNA gene environmental libraries were constructed, and 40 randomly chosen colonies per sample were analyzed for insert 16S rRNA gene sequences. Plasmid DNA containing inserts of the 16S rRNA gene was prepared using a QIAGEN kit. Sequencing reactions were carried out with primers Bac27F and Arch21F and with a DYEnamic ET terminator cycle sequencing ready reaction kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The 16S rRNA gene was sequenced with an ABI 3100 sequencer. Partial sequences were typically ~600 to 700 bp long. Representative bacterial clones and all isolates were sequenced with multiple primers (Bac27F, 925R, and Univ1492R) to obtain nearly full-length (~1,400-bp) sequences of the bacterial 16S rRNA gene. The sequence of 925R was 5′-CCGTCAATTTTRAG TTT. There was an overlap of approximately 200 bases between the members of each pair of primers (e.g., between 27F and 925R). Complementary checking showed that the PCR error frequency was low (<0.05 error per 100 nucleotides). The PCR error was also checked by amplification, cloning, and sequencing of the 16S rRNA gene of *Sulfolobus acidocaldarius* by using the same methods that were used for the samples. Thirty clones of *S. acidocaldarius* were sequenced. Base comparisons between the sequence from the GenBank database and our data indicated that the error frequency for the partial sequencing analysis was approximately 0.05 error per 100 bases.

**T-RFLP analyses.** Terminal restriction fragment length polymorphism (T-RFLP) analyses of the 16S rRNA gene were performed in order to rapidly identify dominant sequences in the samples and to compare the rocks with the drilling fluid samples. The bacterial 16S rRNA gene was amplified from DNA extracts by PCR using the FailSafe PCR enzyme mixture and labeled primers Bac27F-HEX and Univ1492R-TET for bacteria and Arch21F-HEX and 958R-TET for archaea. The T-RFLP PCR and cycling conditions for the bacterial communities were identical to those described above. The primers were 5′ labeled with the phosphoramidite dyes 5-hexachlorofluoresceine and 5-tetrachlorofluorescein, respectively (Operon, Inc., Alameda, CA). PCR replicates were pooled and subjected to agarose gel electrophoresis. The products were cut from each gel lane and purified using a GeneClean Turbo kit as directed by the supplier. Ten microliters of purified 16S rRNA gene sequence was digested in a 20-μl reaction mixture with 3 U of HhaI and HaeIII (New England Biolabs, Beverly, MA) each for 6 h. After 6 h, the restriction enzymes were heat inactivated and precipitated by adding 0.2 μl of 3 M sodium acetate (pH 5.2) and 80 μl of 100% ethanol. The pellet was rinsed with 400 μl 70% ethanol, dried, and then resuspended in 5 μl ultrapure water. The sizes of fluorescently labeled fragments were determined by comparison with internal TAMRA 2500 size standards (Applied Biosystems Inc., Foster City, CA). For T-RFLP analyses, 5 μl restriction digest was denatured in the presence of 5 μl of freshly deionized formamide with 0.2 μl of the TAMRA 2500 marker at 94°C for 3 min. Injection was performed electrokinetically at 15 kV for 5 s, and runs at 15 kV were completed within 45 min in the GeneScan mode with an ABI 310 DNA sequencer. Terminal restriction fragment sizes between 0 and 600 bp with peak heights of ≥100 fluorescence units were determined using the GeneScan analytical software, version 2.02 (Applied Biosystems Inc.).

**Phylogenetic analyses.** Clone sequences were manually checked with the Sequencer program, and secondary structure diagrams and the Chimera Check program were utilized to identify potential chimeras formed during PCR. The sequences obtained were compared to the small-subunit 16S rRNA gene database of Ribosomal Database Project II and GenBank to find the two nearest phylogenetic neighbors and a representative collection of divergent phylogenetic groups, and they were aligned with 16S rRNA gene sequence data from the Ribosomal Database Project and GenBank using the ClustalW multiple-sequence alignment program. The sequences were then manually aligned using the Macclad software. Phylogenetic analyses were performed by application of distance matrix, neighbor-joining, maximum-parsimony, and maximum-likelihood algorithms with PAUP, version 4.0.

**Nucleotide sequence accession numbers.** The sequences determined in this study have been deposited in the GenBank database under accession numbers

TABLE 1. Anion, TOC, and $^{13}C$ isotope compositions of the rocks and drilling fluids[a]

| Sample (depth [m]) | Rock type | F (μg/g) | Cl (μg/g) | $NO_3$ (μg/g) | $SO_4$ (μg/g) | TOC (%) | TOC $\delta^{13}C$ (‰) |
|---|---|---|---|---|---|---|---|
| CCSD_RK529 (529) | Eclogite | 11 | 18 | 6,118 | 126 | 0.003 | −25.6 |
| CCSD_RK730 (730) | Eclogite | 12 | 17 | 15,858 | 1,158 | | |
| CCSD_RK1080 (1,080) | Eclogite | 8 | 13 | 9,173 | 73 | 0.003 | −24.9 |
| CCSD_RK1179 (1,179) | Gneiss | 8 | 6 | 4,237 | 122 | 0.025 | −25.7 |
| CCSD_RK1930 (1,930) | Eclogite | 6 | 13 | 3,264 | 140 | | −26.2 |
| CCSD_RK2026 (2,026) | Amphibolite | 5 | 10 | 9,629 | 45 | 0.02 | −25.4 |
| CCSD_DF730 (730) | | 6 | 502 | 0 | 258 | | |
| CCSD_DF1080 (1,080) | | 6 | 400 | 25 | 161 | 1.07 | −26.6 |
| CCSD_DF1930 (1,930) | | 4 | 187 | 0 | 113 | | |
| CCSD_DF2030 (2,030) | | 4 | 166 | 7 | 226 | 4.68 | −28.6 |

[a] CCSD_DF730, CCSD_DF1080, CCSD_DF1930, and CCSD_DF2030 are the drilling fluid samples from depths corresponding to the depths of rock core samples CCSD_RK730, CCSD_RK1080, CCSD_RK1930, and CCSD_RK2026, respectively.

AY820245 to AY820252 for the isolate sequences, AY820624 to AY820727 for the bacterial clone sequences, and AY820186 to AY820244 for the archaeal clone sequences.

## RESULTS

**Quality control.** Multiple methods revealed that contamination was negligible. The drilling fluids and the rock cores exhibited drastically different Cl concentrations (166 to 502 versus 6 to 18 ppb) and $NO^-_3/SO^{2-}_4$ ratios (<0.5 versus 15 to 200) (Table 1), as determined for the drilling fluid supernatant and the rock leachate, suggesting that the rock cores were not contaminated with the drilling fluid. No 16S rRNA gene of the positive controls (*E. coli* and *S. putrefaciens* CN32) was obtained or amplified by PCR, illustrating that the permeability of the rock cores was sufficiently low so that there was minimal microbial penetration. The TC and TOC concentrations in the rock and drilling mud samples were distinctly different, and their $^{13}C$ values were different (Table 1). The TC content for the rocks was 48- to 64-fold lower than the corresponding values for the drilling fluids from the same depths. The $^{13}C$ value for TC for the rocks was much less negative than the values for the drilling fluids (by −4 to −10‰). The TOC content in the rocks was 200- and 400-fold lower than that in the drilling fluids. The $^{13}C$ value for TOC for the rocks was less negative than that for the drilling fluids, and the difference was statistically significant. These results again suggest that there was minimal or undetectable contamination of the rocks by the drilling fluids.

PLFA analyses of one eclogite sample (CCSD_RK1179 from 1,179 m) revealed the lack of polyunsaturated fatty acids characteristic of eukaryotic organisms. Since the two drilling fluid samples contained a significant proportion of polyunsaturated fatty acids (16 to 28% of the total PLFA), the absence of such biomarkers in the rock provided further support for the lack of contamination from the drilling fluids. Contamination that was possibly introduced during the laboratory procedures was tested using 70% ethanol-sterilized rock cores, and no DNA was detected or amplified. There was no overlap in the microbial communities between the soil and the rocks (see below). The bacterial clone sequences from the rock and the drilling fluid samples were not present in the surface soil sample. Because the circulating drilling fluid "saw" freshly cut rock surfaces and was in contact with subsurface geological fluids from structurally weak shear zones and faults, it was expected that the drilling fluid gradually incorporated microbial communities from the rocks, as well as from geological fluids. These results collectively demonstrated that contamination (if there was any) introduced into the rocks either during sample collection at the drilling site or during the subsequent laboratory analyses could not compromise the in situ microbial abundance in the rocks.

**Petrophysical and geochemical analyses.** The pH values of the drilling fluid samples ranged from 9 to 10.5. The fluorine and sulfate concentrations were similar for the rocks and the drilling fluids. The chloride concentration in the drilling fluids was 16- to 31-fold higher than that in the rocks, but the nitrate concentration in the rocks was much higher than that in the drilling fluids (>366- to 1,376-fold) (Table 1). The high nitrate concentration was likely from saline fluid inclusions in the rocks (37). This inference was supported by the fact that the eclogite samples, which contained garnet and pyroxene, two dominant host minerals for fluid inclusions, had higher concentrations of nitrate than amphibolite (CCSD_RK2026). The eclogite CCSD_RK1930 sample was enriched in white mica and did not have abundant garnet and pyroxene, possibly accounting for its low nitrate concentration. Similar observations were made by Onstott et al. (27), and these authors ascribed the high concentration of nitrate to fluid inclusions in the crushed quartzite and the Carbon Leader samples. The TOC content ranged from 0.003 to 0.03% for the rocks, with corresponding $^{13}C$ values of −24.9 to −26.2 per mil, and from 1 to 5% for the drilling fluids, with corresponding $^{13}C$ values of −26.6 to −28.6 per mil.

The rock samples ranged in porosity from 1 to 3.5% and had a permeability of ~0.5 mDarcy. The rocks were eclogite and amphibolite (Table 2), and high-pressure minerals such as coesite (in CCSD_RK730) and kyanite (in CCSD_RK1080) were present, as determined by X-ray diffraction and electron microprobe analyses. Whole rock chemical analyses revealed that the rock samples contained significant amounts of $Fe_2O_3$ and FeO. Electron microprobe analyses showed that most of $Fe_2O_3$ and FeO were in garnet, pyroxene, and ilmenite. Garnet contained almadine-grossular-pyrope components, and pyroxene belonged to omphacite. Both garnet and pyroxene contained some $P_2O_5$ (Table 2). Apatite was present because the $P_2O_5$ concentration in garnet and pyroxene was lower than that in the whole rock, and there were no other $P_2O_5$-bearing minerals. $H_2O$ was present as fluid inclusions (37) or as structural

TABLE 2. Chemical compositions of the rock samples from the CCSD project[a]

| Sample (depth [m]) | Rock type | Rock or mineral composition | $Fe_2O_3$ (%) | FeO (%) | $P_2O_5$ (%) | $H_2O$ (%) | $CO_2$ (%) |
|---|---|---|---|---|---|---|---|
| CCSD_RK529 (529) | Eclogite | Whole rock | 4.8 | 15.8 | 0.24 | 0.42 | <0.01 |
| | | Garnet | 0.7 ± 0.2 | 23.4 ± 0.2 | 0.10 ± 0.004 | | |
| | | Pyroxene | 14.3 ± 2.6 | 6.3 ± 2.6 | 0.08 ± 0.01 | | |
| CCSD_RK730 (730) | Eclogite | Whole rock | 2.3 | 9.3 | 0.04 | 0.48 | 0.18 |
| | | Garnet | 0.6 ± 0.3 | 20.8 ± 0.2 | 0.09 ± 0.002 | | |
| | | Pyroxene | 4.9 ± 4.2 | 3.5 ± 2.7 | 0.10 ± 0.03 | | |
| CCSD_RK1080 (1,080) | Eclogite | Whole rock | 4.5 | 4.3 | 0.42 | 0.63 | 0.15 |
| CCSD_RK1179 (1,179) | Gneiss | Whole rock | 1.2 | 0.6 | 0.02 | 0.24 | 0.52 |
| CCSD_RK1930 (1,930) | Eclogite | Whole rock | 3.5 | 7.4 | 0.15 | 1.26 | 0.18 |
| CCSD_RK2026 (2,026) | Amphibolite | Whole rock | 3.8 | 6.5 | 0.34 | 1.40 | 0.09 |

[a] CCSD_RK529 is an eclogite sample containing pyrite, rutile, garnet, pyroxene, and ilmenite; CCSD_RK730 is an eclogite sample containing cosite, rutile, garnet, and pyroxene; CCSD_RK1080 is a slightly retrograde eclogite sample containing kyanite, rutile, epidote, and garnet; CCSD_RK1930 is an eclogite sample containing Si-rich white mica; and CCSD_RK2026 is an amphibolite sample containing amphibole. The composition of the whole rock was determined by the wet chemistry method; the composition of individual minerals was determined by electron microprobe analyses. The compositions of the individual minerals are averages for six to eight spot analyses. The totals were 98.91 to 100.09% for the whole rock analyses and 95 to 101% for the individual minerals in CCSD_RK529 but 99 to 101% for the minerals in CCSD_RK730.

hydroxyl groups in alteration minerals. $CO_2$ probably was present as carbonate and organic carbon and as $CO_2$ gas in fluid inclusions (37). Scanning electron microscopy images showed that fractures were primarily present within garnet and pyroxene (Fig. 2), two main minerals containing various fluid and gas fluid inclusions.

Abundant fluid and gas inclusions were present inside minerals of the UHP rocks (Fig. 3). There were four types of inclusions (37). Type I inclusions were aqueous inclusions with high-salinity $CaCl_2$-NaCl-$H_2O$ fluid (75% $H_2O$, 17% NaCl, and 8% $CaCl_2$, by weight), and they occurred in garnet and pyroxene of eclogite. These inclusions typically ranged in size from 12 to 40 μm and were inferred to be metamorphic in origin. Type II inclusions contained mixed $H_2O$-$CO_2$ (+$N_2$)-NaCl solids (45 to 70% $H_2O$, 5 to 30% $CO_2$, and 1 to 25% NaCl, by moles percent); they occurred in quartz and kyanite of eclogite, and the sizes were similar to the sizes of type I inclusions. Some inclusions contained a small amount of $N_2$. Shen et al. (37) inferred that type II inclusions were introduced into the UHP rocks during exhumation and later retrogression. Type III inclusions contained aqueous solutions having low to medium salinity in garnet, pyroxene, and apatite of eclogite. Type IV inclusions contained carbonates having medium to low salinity. Type III and IV inclusions (Fig. 3, upper left) were distributed along intragranular fractures of garnet and formed when the UHP rocks were uplifted to the crust at low temperatures and pressures.

**Biomass determination by direct counting and PLFA analysis.** Total counts of microbial cells in the rocks were determined by acridine orange direct counting. The data indicated that the number of cells ranged from $5.2 \times 10^3$ to $2.4 \times 10^4$ cells per g (dry weight) of rock powder. This level of biomass was comparable to that reported for quartzite from deep mines in South Africa (27). PLFA represent viable microbial populations (42). The PLFA concentration in the eclogite sample (CCSD_RK1179) was 4.9 pmol/g, which was equivalent to $9.8 \times 10^4$ cells/g (Microbial Insights, Inc., personal communica-

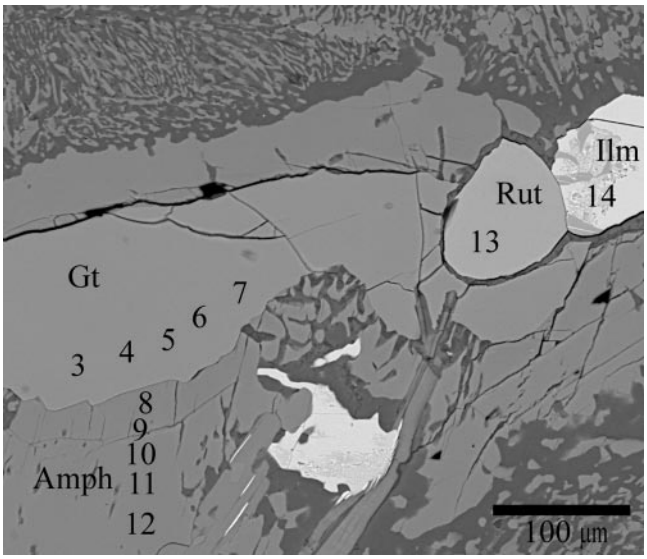



FIG. 2. Back-scattered electron image showing fractures in garnet and pyroxene. The numbers are spots used for obtaining quantitative chemical composition by electron microprobe analyses. Gt, garnet; Rut, rutile; Ilm, ilmenite;Amph, amphibole.

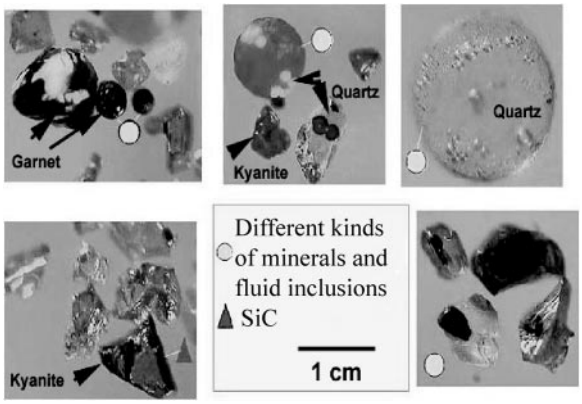



FIG. 3. Optical micrograph showing various mineral and fluid inclusions in the UHP rocks. The upper left and lower right images show inclusions in garnet and pyroxene. The upper middle and upper right images show inclusions in quartz (colorless to gray) and kyanite. The lower left image shows inclusions in kyanite. All the host minerals are from eclogite.

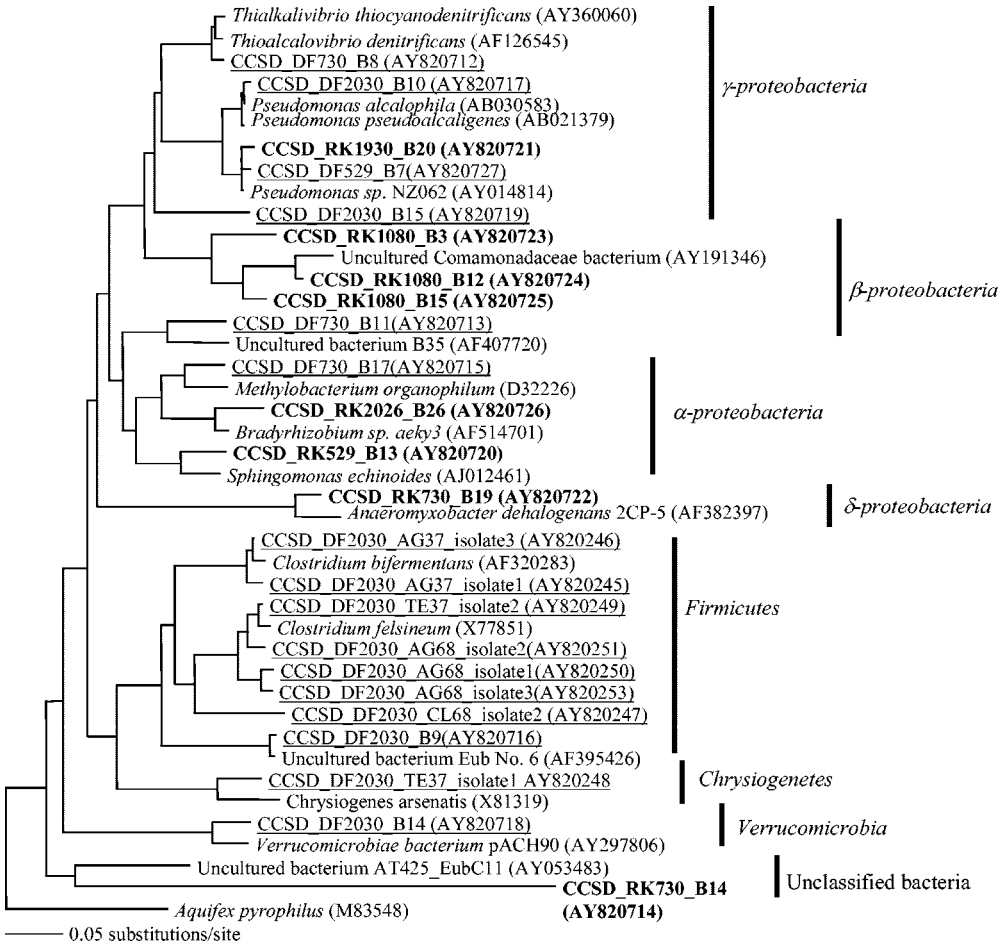



FIG. 4. Neighbor-joining tree of nearly full-length sequences (~1,400 bp) of isolates and representative bacterial clone sequences. As an example, the CCSD_DF2030_AG37_isolate1 designation indicates the following: CCSD, Chinese continental scientific drilling; DF2030, drilling fluid from a depth of 2,030 m; AG37, AG medium with an incubation temperature of 37°C; isolate1, isolate number. Thus, this isolate was obtained from CCSD Project drilling fluid from a depth of 2,030 m, using AG medium at 37°C.

tion). The prokaryotic profiles were as follows: br15:0d, 12%; 16:0, 22%; br16:0a, 13%; br16:1a, 22%; 18:1ω9c, 9%; 18:0, 8%; and 2Me18:0, 18%. The branched saturated acids are characteristic of heterotrophic bacteria, such as sulfate- and metal-reducing bacteria, and may indicate the presence of such bacteria in the sample. The biomass values for the two drilling fluid samples were $3.5 \times 10^8$ and $7.5 \times 10^8$ cells/g for CCSD_DF730 and CCSD_DF1080, respectively. The community structure analyses of the two drilling fluids indicated that *Proteobacteria* and normal saturated PLFA were the prominent constituents of the two drilling fluid samples. *Firmicutes* or *Clostridium*-like fermenting bacteria (terminally branched PLFA) were also present in the two drilling fluids (20% and 10% of the total PLFA for CCSD_DF730 and CCSD_DF1080, respectively). The biomarker for sulfate-reducing bacteria was present in the drilling fluids, and the levels ranged from 9% (CCSD_DF730) to 17% (CCSD_DF1080) of the total PLFA. An anaerobic metal reducer constituted a minor component (5% and 4% for CCSD_DF730 and CCSD_DF1080, respectively).

**Enrichment cultures.** In the rock enrichments at an incubation temperature of 37°C in M1 medium, there were visible cell growth and formation of a black precipitate, suggesting thermophilic Fe(III) reduction activity. Magnetic testing verified that the precipitate was magnetic, most likely magnetite. There was no evident Fe(III) reduction either in M1 medium at other incubation temperatures or in FWA-Fe(III) medium. In the enrichments for drilling fluid sample CCSD_DF2030 (2,030 m) with TE medium, reduction of Fe(III) in oxyhydroxide was observed, and Fe(II) production occurred, as measured by the Ferrozine assay. Several isolates were obtained from the drilling fluid sample. Two isolates obtained from AG medium at 37°C (CCSD_DF2030_AG37_isolate1 and CCSD_DF2030_AG37_isolate2) were closely related (96 to 98% similarity) to *Clostridium bifermentans* (Fig. 4), an organism capable of using aromatic compounds as electron acceptors for fermentation (6). One isolate from TE medium at an incubation temperature of 37°C (CCSD_DF2030_TE37_isolate1) and one isolate from AG medium at an incubation temperature of 68°C (CCSD_DF2030_AG68_isolate1) were 96 to 98% similar to *Clostridium felsineum*. Two other isolates from AG medium at an incubation temperature of 68°C (CCSD_DF2030_AG68_isolate2 and CCSD_DF2030_AG68_isolate3) were only moderately related (94% similarity) to *C. felsineum*. One isolate obtained from CL medium at an incuba-

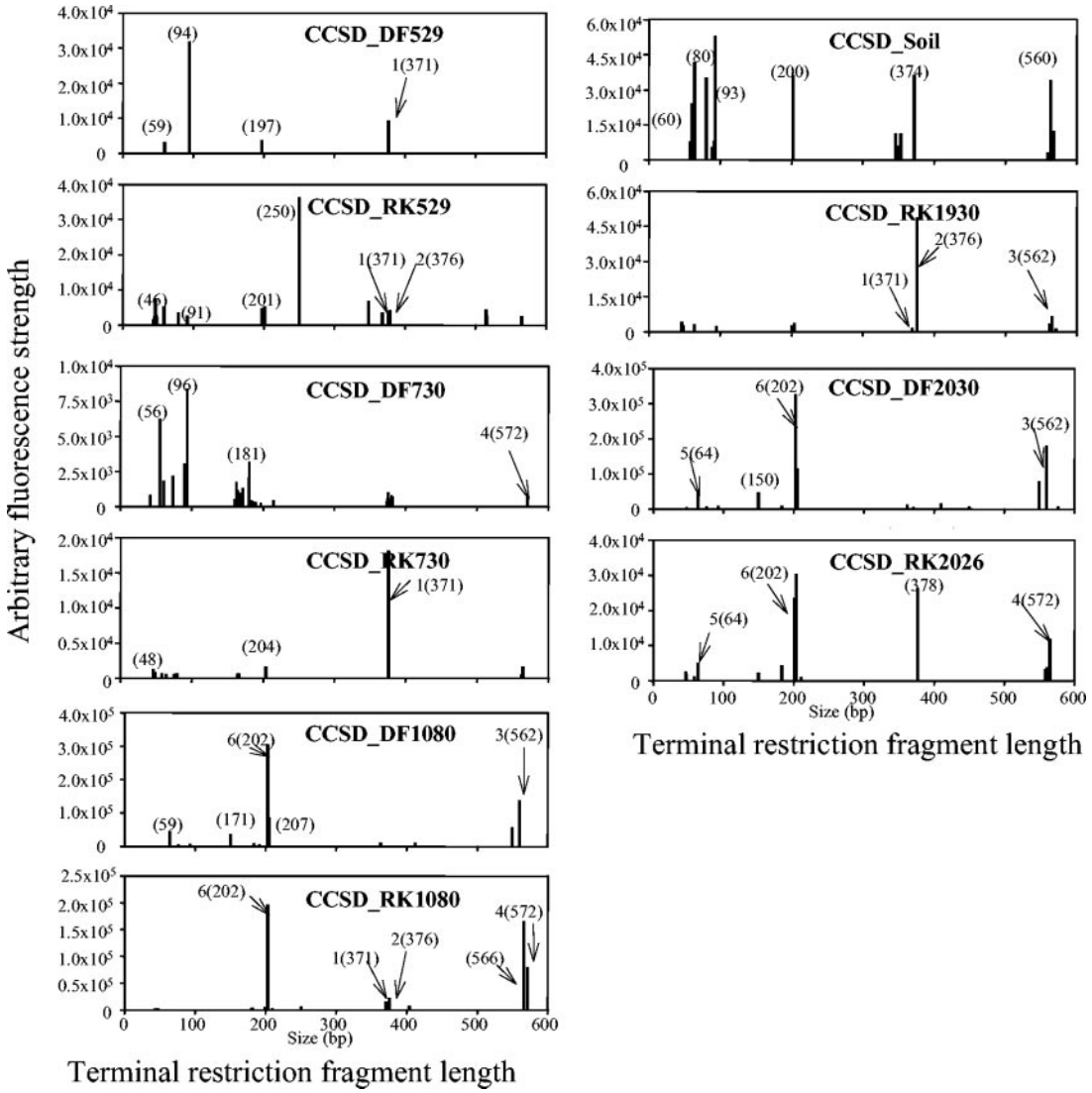
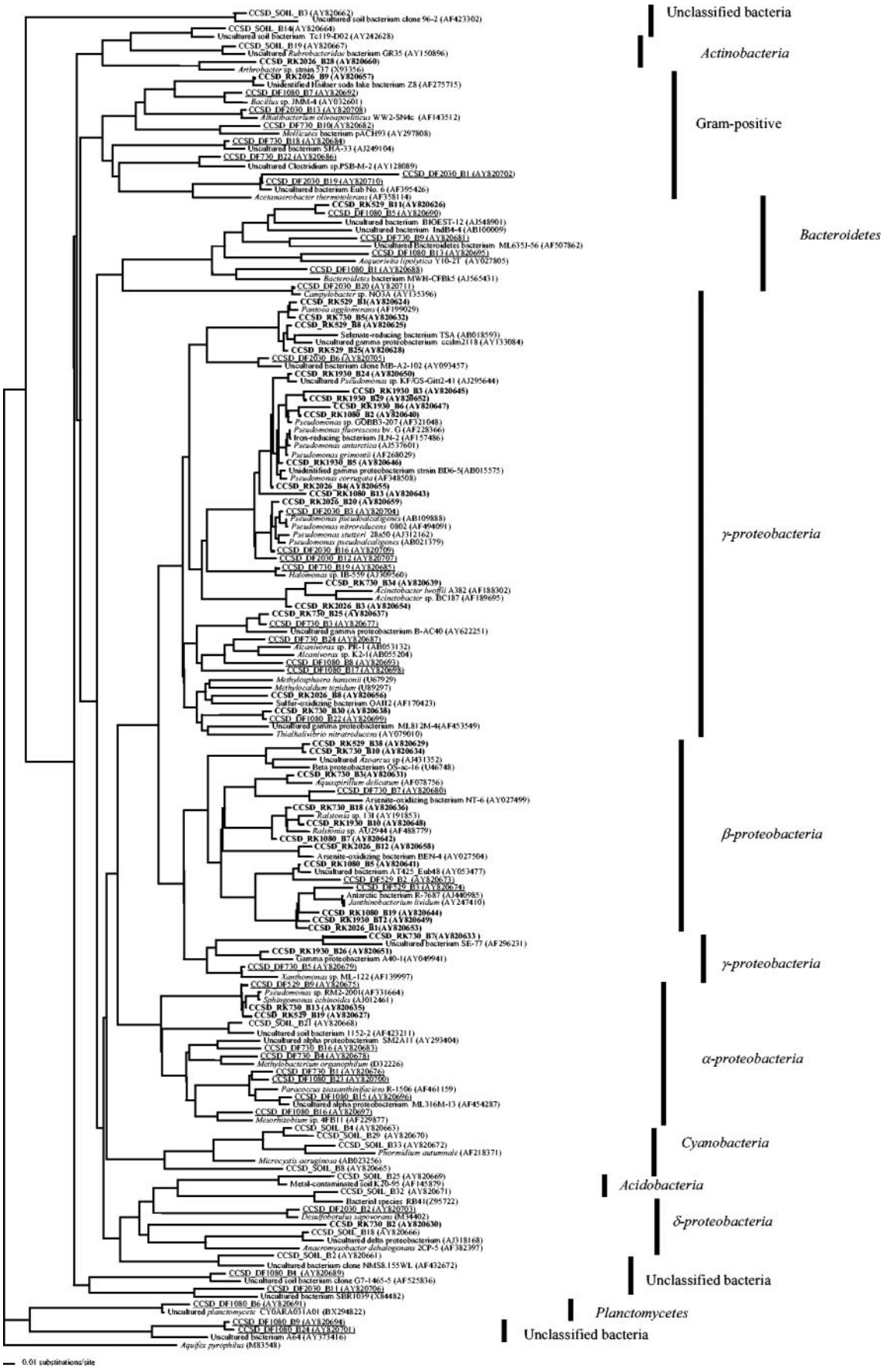



FIG. 5. Typical electropherograms of bacterial T-RFLPs generated from the rRNA gene with a labeled forward primer and enzyme HhaI digests for the rock, drilling fluid, and surface soil samples. Another set of electropherograms of bacterial T-RFLPs generated from the rRNA gene was obtained with a labeled reverse primer, but these electropherograms are not shown. The numbers above or next to the peaks indicate major ribotypes common to all the samples, and the unlabeled peaks indicate novel ribotypes unique to a sample. For example, none of the peaks for the surface soil sample is labeled, indicating that all the peaks (ribotypes) are unique to the soil sample. The numbers in parentheses are fragment sizes in base pairs.

tion temperature of 68°C (CCSD_DF2030_CL68_isolate1) was not related to any cultured bacterium or environmental clone at the genus level (i.e., 93 to 95% similarity). One isolate from TE medium at an incubation temperature of 37°C (CCSD_DF2030_TE37_isolate2) was only moderately related (91% similarity) to *Chrysiogenes arsenatis*, an organism that reduces arsenate to arsenite.

**T-RFLP analyses.** T-RFLP analyses (Fig. 5) showed that although some terminal restriction fragments (ribotypes) were common to both the rocks and the corresponding drill fluid samples (for example, the terminal restriction fragment at 371 bp from CCSD_RK529 and the corresponding drilling fluid), in general there was a significant distinction between the rocks and the drilling fluids. More importantly, the T-RFLP pattern for the surface soil was distinct from those for all the rocks and the drilling fluids. There were approximately five or six major terminal restriction fragments (ribotypes) in each rock.

**Bacterial diversity. (i) CCSD_RK529 (529 m).** The DNA sequences from the CCSD_RK529 sample clustered into four major lineages: the α-, β-, and γ-subdivisions of the *Proteobacteria* and the *Cytophaga-Flexibacter-Bacteroides* group (Fig. 6 and Table 3). One group of clones was closely related to a cultured organism, *Pantoea agglomerans*, an anaerobic Fe(III)-, Mn(IV)-, and Cr(VI)-reducing bacterium (9). Another group was related to a selenate-reducing bacterium, strain TSA. One sequence (B19) was closely related and another sequence (B13) was remotely related to *Sphingomonas echinoides*. The genus *Sphingomonas* has been isolated from subsurface sediments, and species in this genus are generally mesophilic, heterotrophic, and gram negative (1). A sequence



related to *Azoarcus* sp. was present. The species of *Azoarcus* have been shown to anaerobically reduce nitrate and to degrade aromatic compounds (25). Three sequences were related to a bacterial clone from deep-sea hydrothermal systems (uncultured bacterium IndB4-4) (GenBank description).

One group of clones from the drilling fluid CCSD_DF529 sample (four clones) was closely related (97% similarity) to *Janthinobacterium lividum* in the β-*Proteobacteria* (Fig. 6 and Table 3), a facultative nitrate-reducing bacterium typically present in soil and water. This organism has been isolated from estuary sediments and Lake Fryxell, McMurdo Dry Valleys, Antarctica (5), in both aerobic and anaerobic enrichments. One sequence (B2) was related to a clone from Gulf of Mexico gas hydrates (20). The surface soil clone types were very diverse (Fig. 6). Seventeen nonchimeric sequences belonged to the α-, δ, and, γ-subdivisions of the *Proteobacteria*, *Actinobacteria*, and *Cyanobacteria*. Phylogenetic analyses demonstrated that there was no overlap between the surface soil clone library and either the drilling fluid CCSD_DF529 or rock CCSD_RK529 clone library (Fig. 6).

**(ii) CCSD_RK730 (730 m).** Phylogenetic analyses showed that the 16S rRNA gene sequences from the CCSD_RK730 sample clustered into four major lineages of bacteria, the α-, β-, γ-, and, δ-subdivisions of the *Proteobacteria*, along with some unidentified sequences (Fig. 6 and Table 3). In addition to the clone type closely related to *P. agglomerans*, the second most abundant group of clones was closely related to *Aquaspirillum delicatum*. Most species in this genus have been isolated from freshwater. Some species can fix nitrogen. *Aquaspirillum autotrophicum* is an $H_2$-oxidizing, facultatively autotrophic bacterium. Some strains are aerobic and tolerate low salinity (<3% NaCl). Another group of clones was almost identical to *Ralstonia* sp., a denitrifying facultative microbe resistant to heavy metals and capable of degrading hydrocarbons at 30°C (21). Other clone sequences showed similarity to *Pseudomonas* sp. (99%), *Stenotrophomonas maltophilia* or uncultured bacterium SE-77 (98%), *Acinetobacter lwoffii* (95%), or *Thioalkalivibrio nitratireducens* (90%). *T. nitratireducens* is an anaerobic obligately chemolithoautotrophic sulfur-oxidizing bacterium from soda lakes. These organisms use sulfide as the electron donor and nitrate or nitrous oxide as electron acceptors under alkaline conditions (38). One sequence was remotely related to *Anaeromyxobacter dehalogenans*, an anaerobic organism that reduces nitrate to ammonium (35).

The 16S rRNA gene sequences from the drilling fluid CCSD_DF730 sample fell into the α, β-, and γ-*Proteobacteria*, the low-G+C-content gram-positive bacteria, the *Bacteroidetes*, and the gram-positive bacteria (Fig. 6 and Table 3). Five sequences clustered with the α-*Proteobacteria* group with similarity to *Paracoccus zeaxanthinifaciens* and *Methylobacterium organophilum*. The species of *Paracoccus* occur in many terrestrial environments and have a wide range of metabolic modes (autotrophy, methylotrophy, and mixotrophy). Many species are capable of denitrification under anoxic conditions, with nitrate as the respiratory oxidant and organic compounds as the carbon and energy sources (17). *M. organophilum* was isolated from the metalimnion of Lake Mendota in the United States and is an aerobic, gram-negative, rod-shaped bacterium that is able to grow on compounds more reduced than carbon dioxide as sole carbon and energy sources (12). Two clone sequences in the β-*Proteobacteria* were closely related to new arsenite-oxidizing bacteria isolated from Australian gold mining environments. The sequences of the γ-*Proteobacteria* were closely related to *Xanthomonas* sp., *Halomonas* sp., and *Alcanivorax* sp. (Fig. 6 and Table 3). *Xanthomonas* sp. strain ML-122 is a novel alkalohalophile isolated from Mono Lake sediment in California. All of the members of the genus *Halomonas* are gram-negative rods that exhibit extreme tolerance to NaCl. *Halomonas* reduces nitrate to nitrite, and most of the species can grow under anaerobic conditions in the presence of nitrate (41). The species of *Alcanivorax* are capable of anaerobic growth with nitrate. Members of this genus are moderately halophilic, and the optimal NaCl concentration for growth is 3 to 10%. The optimum temperature is 20 to 30°C (11). The two sequences in the *Bacteroidetes* were remotely related to two environmental clones from alkaline, hypersaline, and currently meromictic Mono Lake in California (14). The clone sequences in the gram-positive bacterial division showed low similarity to any known sequences in the GenBank database.

**(iii) CCSD_RK1080 (1,080 m).** The 16S rRNA gene sequences in the CCSD_RK1080 sample fell into two lineages of bacteria, the β- and γ-*Proteobacteria* (Fig. 6 and Table 3). In the β-*Proteobacteria*, one group of clone sequences was closely related to the Antarctic bacterium R-7687 and *J. lividum.* The Antarctic bacterium R-7687 was obtained from microbial mats of Antarctic lakes (40). Sequences similar to the sequences of *Delftia acidovorans* and uncultured bacterium AT425_Eub48 from the Gulf of Mexico gas hydrate and the Nankai Trough were also present. *D. acidovorans* is aerobic and can reduce nitrate to nitrite. Four sequences in the γ-*Proteobacteria* were related to species of *Pseudomonas*.

Twenty-three clone sequences from the CCSD_DF1080 drilling fluid sample fell into five major lineages of bacteria, the α- and γ-*Proteobacteria*, *Planctomycetes*, *Bacteroidetes*, and gram-positive bacteria (Fig. 6). In addition to *Paracoccus* and *Alcanivorax*, there were new clone types. The most abundant clone type was not closely related to any known cultured bacteria or environmental clones. It was remotely related to *Bacteroidetes* strain MWH-CFBk5, which may have come from makeup water. Two sequences (B5 and B19) were moderately related to anaerobic bacterial clone BIOEST-12 from a microbial film for

FIG. 6. Phylogenetic relationships of representative phylotypes of bacterial 16S rRNA gene sequences as determined by the neighbor-joining method. Scale bar = 0.01 nucleotide substitution per site. Sequences for the rock samples are indicated by uppercase boldface type, sequences for the drilling fluid samples are underlined, and sequences from the surface soil sample are indicated by the prefix CCSD_SOIL. The remaining sequences were reference sequences obtained from the Ribosomal Database Project or GenBank. Phyla were determined by using the classification in *Bergey's Manual of Systematic Bacteriology* (10). *Aquifex pyrophilus* is used as an outgroup. GenBank accession numbers are in parentheses. The results of a complete phylogenetic bacterial 16S rRNA gene clone type analysis are shown in Table 3.

TABLE 3. Phylogenetic bacterial rRNA gene clone type analysis

| Sample (depth [m]) | Phylogenetic group | Clone type | Related organism or clone | % Similarity | No. of related clones/total no. |
|---|---|---|---|---|---|
| CCSD_RK529 (529) | α-*Proteobacteria* | B19 | *Sphingomonas echinoides* | 93–100 | 2/16 |
| | β-*Proteobacteria* | B38 | *Azoarcus* | 92 | 1/16 |
| | γ-*Proteobacteria* | B1 | *Pantoea agglomerans* | 97–99 | 5/16 |
| | | B8 | Selenate-reducing bacterium TSA | 97 | 3/16 |
| | | B25 | Uncultured gamma proteobacterium clone ccslm2118 | 96–99 | 2/16 |
| | *Bacteroidetes* | B11 | Uncultured bacterium IndB4-4 | 88 | 3/16 |
| CCSD_RK730 (730) | α-*Proteobacteria* | B13 | *Pseudomonas* sp. | 99 | 1/28 |
| | β-*Proteobacteria* | B3 | *Aquaspirillum delicatum* | 96–97 | 5/28 |
| | | B10 | Uncultured *Azoarcus* or β-proteobacterium OS-ac-16 (U46748) | 92–93 | 2/28 |
| | | B18 | *Ralstonia* sp. strain 131 | 99 | 3/28 |
| | γ-*Proteobacteria* | B7 | Uncultured bacterium SE-77 or *Stenotrophomonas maltophilia* | 92–96 | 2/28 |
| | | B5 | *Pantoea agglomerans* | 97–100 | 8/28 |
| | | B25 | *Thioalkalivibrio nitratireducens* | 88 | 2/28 |
| | | B34 | *Acinetobacter Iwoffii* | 95 | 1/28 |
| | | B30 | Uncultured gamma proteobacterium clone ML812M-4 | 88 | 1/28 |
| | δ-*Proteobacteria* | B2 | *Anaeromyxobacter dehalogenans* | 78–79 | 2/28 |
| CCSD_RK1080 (1,080) | β-*Proteobacteria* | B7 | *Ralstonia* | 97 | 1/15 |
| | | B19 | Antarctic bacterium or *Janthinobacterium lividum* | 88–99 | 5/15 |
| | | B5 | Uncultured bacterium AT425_Eub48 | 92–97 | 3/15 |
| | γ-*Proteobacteria* | B2 | *Pseudomonas fluorescens* | 98 | 2/15 |
| | | B13 | *Pseudomonas* sp. strain GOBB3-207 | 88–89 | 2/15 |
| CCSD_RK1930 (1,930) | β-*Proteobacteria* | B12 | *Janthinobacterium lividum* or Antarctic bacterium | 98–99 | 3/21 |
| | | B10 | *Ralstonia* sp. | 98 | 1/21 |
| | | B5 | Iron-reducing bacterium JLN-2 | 98–99 | 2/21 |
| | γ-*Proteobacteria* | B6 | Unidentified gamma-proteobacterium strain BD6-5 or BD4-3 | 97–99 | 4/21 |
| | | B3 | *Pseudomonas grimontii* | 94–98 | 7/21 |
| | | B26 | γ-Proteobacterium A40-l | 95 | 1/21 |
| | | B29 | *Pseudomonas corrugata* | 98 | 1/21 |
| | | B24 | Uncultured bacterium KF/GS-JG36-13 | 97 | 1/21 |
| CCSD_RK2026 (2,026) | β-*Proteobacteria* | B1 | *Janthinobacterium lividum* | 99–100 | 4/22 |
| | | B12 | Arsenite-oxidizing bacterium BEN-4 | 99 | 1/22 |
| | γ-*Proteobacteria* | B4 | *Pseudomonas antarctica* | 99 | 2/22 |
| | | B8 | *Methylocaldum tepidum* | 88–90 | 5/22 |
| | | B20 | *Pseudomonas pseudoalcaligenes* | 97–99 | 6/22 |
| | | B3 | *Acinetobacter* sp. | 98 | 1/22 |
| | *Actinobacteria* | B28 | *Arthrobacter* sp. | 91 | 1/22 |
| | Gram-positive bacteria | B9 | Unidentified Hailaer soda lake bacterium | 99 | 1/22 |
| CCSD_DF529 (529) | α-*Proteobacteria* | B9 | *Sphingomonas echinoides* | 98 | 1/9 |
| | β-*Proteobacteria* | B2 | Uncultured bacterium AT425_Eub48 | 87–94 | 1/9 |
| | | B3 | *Janthinobacterium lividum* | 95–97 | 4/9 |
| CCSD_DF730 (730) | α-*Proteobacteria* | B1 | *Paracoccus zeaxanthinifaciens* | 96 | 2/20 |
| | | B16 | Uncultured alpha-proteobacterium clone SM2A11 | 92 | 1/20 |
| | | B-4 | *Methylobacterium organophilum* | 94 | 2/20 |
| | β-*Proteobacteria* | B7 | Arsenite-oxidizing bacterium NT-6 | 97 | 2/20 |
| | γ-*Proteobacteria* | B3 | Uncultured γ-proteobacterium | 93 | 1/20 |
| | | B19 | *Halomonas* sp. strain IB-559 | 99 | 1/20 |
| | | B5 | *Xanthomonas* sp. strain ML-122 | 96 | 1/20 |
| | | B24 | *Alcanivorax* sp. strain PR-1 | 92 | 1/20 |
| | *Bacteroidetes* | B9 | Uncultured *Bacteroidetes* bacterium clone ML635J-56 | 88–91 | 2/20 |
| | Gram-positive bacteria | B18 | Uncultured bacterium SHA-33 | 97 | 1/20 |
| | | B22 | Uncultured *Clostridium* sp. clone PSB-M-2 | 94 | 1/20 |
| | | B10 | *Mollicutes* bacterium pACH93 | 91 | 1/20 |
| CCSD_DF1080 (1,080) | *Planctomycetes* | B6 | Uncultured planctomycete CY0ARA031A01 | 97 | 1/23 |
| | *Bacteroidetes* | B1 | *Bacteroidetes* bacterium MWH-CFBk5 | 85–90 | 6/23 |
| | | B13 | *Aequorivita lipolytica* Y10-2T | 89–90 | 2/23 |
| | | B4 | Uncultured soil bacterium clone | 96 | 1/23 |
| | | B5 | Uncultured bacterium clone BIOEST-12 | 90 | 2/23 |
| | | B11 | *Sporocytophaga myxococcoides* | 90 | 1/23 |

*Continued on facing page*

TABLE 3—*Continued*

| Sample (depth [m]) | Phylogenetic group | Clone type | Related organism or clone | % Similarity | No. of related clones/total no. |
|---|---|---|---|---|---|
| | Gram-positive bacteria | B7 | *Bacillus* sp. strain JMM-4 | 96 | 1/23 |
| | α-*Proteobacteria* | B23 | *Paracoccus zeaxanthinifaciens* | 94 | 1/23 |
| | | B15 | Uncultured alpha-proteobacterium clone ML316M-13 | 98 | 1/23 |
| | | B16 | *Mesorhizobium* sp. strain 4FB11 | 93 | 1/23 |
| | γ-*Proteobacteria* | B8 | *Alcanivorax* sp. strain K2-1 | 90 | 1/23 |
| | | B22 | Sulfur-oxidizing bacterium OA112 | 88 | 1/23 |
| | | B17 | *Methylosphaera hansonii* U67929 | 85 | 1/23 |
| | | B14 | Uncultured proteobacterium clone | 85 | 1/23 |
| | Unidentified | B9 | Unidentified bacterium clone K2-S7 | 86–87 | 2/23 |
| CCSD_DF2030 (2,030) | *Candidatus* | B11 | Uncultured bacterium SBR1039 | 88 | 1/19 |
| | γ-*Proteobacteria* | B3 | *Pseudomonas nitroreducens* | 97–99 | 4/19 |
| | | B16 | *Pseudomonas pseudoalcaligenes* | 100 | 2/19 |
| | | B12 | *Pseudomonas stutzeri* strain 28a50 | 95 | 1/19 |
| | | B6 | Uncultured bacterium clone MB-A2-102 | 95–96 | 4/19 |
| | δ-*Proteobacteria* | B2 | *Desulfobotulus sapovorans* M34402 | 97 | 2/19 |
| | | B1 | Uncultured bacterium Eub No. 6 | 98 | 1/19 |
| | Gram-positive bacteria | B19 | *Acetanerobacter thermotolerans* | 89 | 1/19 |
| | | B13 | *Alkalibacterium olivoapovliticus* | 97 | 1/19 |
| | ε-*Proteobacteria* | B20 | *Campylobacter* sp. strain NO3A | 98 | 1/19 |

anaerobic treatment of sulfurous effluents. Two sequences (B13 and B20) were related to *Aequorivita lipolytica*, a sublithic bacterium associated with Antarctic quartz stones (4). Two sequences (B15 and B23) in the α-*Proteobacteria* were closely related to a clone from hypersaline and alkaline Mono Lake in California and halobenzoate-degrading denitrifying bacteria, respectively. One sequence (B16) was related to *Mesorhizobium* sp. strain 4FB11, an anaerobic nitrate reducer. In the γ-*Proteobacteria* subdivision, one sequence (B22) showed a low level of identity (88%) to a sulfur-oxidizing bacterium from a shallow water hydrothermal vent. One sequence (B17) was remotely related to a methanotroph (*Methylosphaera hansonii*). The sequence in the gram-positive lineage (B7) showed 96% similarity to an arsenate-respiring bacterium, *Bacillus* sp. strain JMM-4. The phylogenetic positions of two sequences (B9 and B24) could not be determined, and they were moderately related to an unidentified bacterium in deep-sea sediment from a Western Pacific warm pool (GenBank description).

**(iv) CCSD_RK_1930 (1,930 m).** The diversity was much lower in the CCSD_RK_1930 sample. All clone sequences belonged to the β- and γ-*Proteobacteria* (Fig. 6 and Table 3). One group of clones was closely related to a sequence (unidentified γ-proteobacterial strain BD6-5 or BD4-3) from deep-sea sediments (22) or *Pseudomonas* sp. strain NZ062. Another group of clones was closely related to an iron-reducing bacterium, strain JLN-2. Still another group of clones was closely related to *J. lividum* or an Antarctic bacterium, strain R7687. One sequence was related to *Ralstonia* sp. One sequence was closely related to γ-proteobacterial strain A40-1, an aciduric proteobacterium isolated from pH 2.9 soil (8). Eight of 21 clone sequences were closely related to *Pseudomonas*, which included *Pseudomonas* sp. strain QSSC1-9, *Pseudomonas grimontii*, *Pseudomonas fluorescens* strain ATCC 13525, *Pseudomonas corrugata*, and *Pseudomonas* sp. strain KF/GS-Gitt2-41.

**(v) CCSD_RK2026 (2,026 m).** Twenty-two 16S rRNA gene sequences from the CCSD_RK2026 sample phylogenetically clustered in the β- and γ-*Proteobacteria*, gram-positive bacteria, and *Actinobacteria* (Fig. 6 and Table 3). In addition to the sequences present in CCSD_RK1930, there were new clone types. A major group (six sequences) was closely related to *Pseudomonas pseudoalcaligenes*. *P. pseudoalcaligenes* is metal resistant and reduces nitrate to $N_2$. Another major new type (five sequences) was moderately related to *Methylocaldum tepidum*, a thermophilic methanotrophic bacterium (2). Other sequences were related to *Pseudomonas antarctica* or the iron-reducing bacterium strain JLN-2, *Acinetobacter*, *Arthrobacter*, and a clone from a soda lake. *P. antarctica* is an iron-reducing bacterium isolated from Antarctic lakes. Some *Arthrobacter* species are capable of dinitrogen fixation. One clone sequence (B9) was closely related to the sequence of an unidentified Hailaer soda lake bacterium, strain Z8 (GenBank description).

Nineteen sequences from the CCSD_DF2030 drilling fluid sample fell into five major lineages of bacteria, the ε-, δ-, and γ-*Proteobacteria*, the *Candidatus* group, and the gram-positive bacteria. Two groups were dominant, and both of them were in the γ-*Proteobacteria*; type B3 was closely related to *Pseudomonas nitroreducens*, and type B6 was closely related to uncultured bacterial clone MB-A2-102 from the methane hydrate-bearing deep marine sediments in the forearc basin of the Nankai Trough near Japan (32). Other clone sequences were closely related to *P. pseudoalcaligenes*, *Desulfobotulus sapovorans*, and uncultured bacterium Eub No. 6. Other clone sequences were related to *Campylobacter* sp. strain NO3A, a nitrate-reducing, sulfide-oxidizing bacterium, *Acetanaerobacter thermotolerans*, a strictly anaerobic, moderately thermophilic acetogen and sulfur reducer, and *Alkalibacterium olivoapovliticus*, a motile, psychrotolerant, halotolerant, facultatively anaerobic bacterium with a pH optimum of 9 to 10.

**Archaeal diversity.** The archaeal diversity was much lower, and all archaeal sequences formed four groups within the *Crenarchaeota* (Fig. 7). All sequences from the CCSD_DF529 sample (26 sequences) and two sequences from the CCSD_DF2030 sample formed the first group and were 93 to 98%

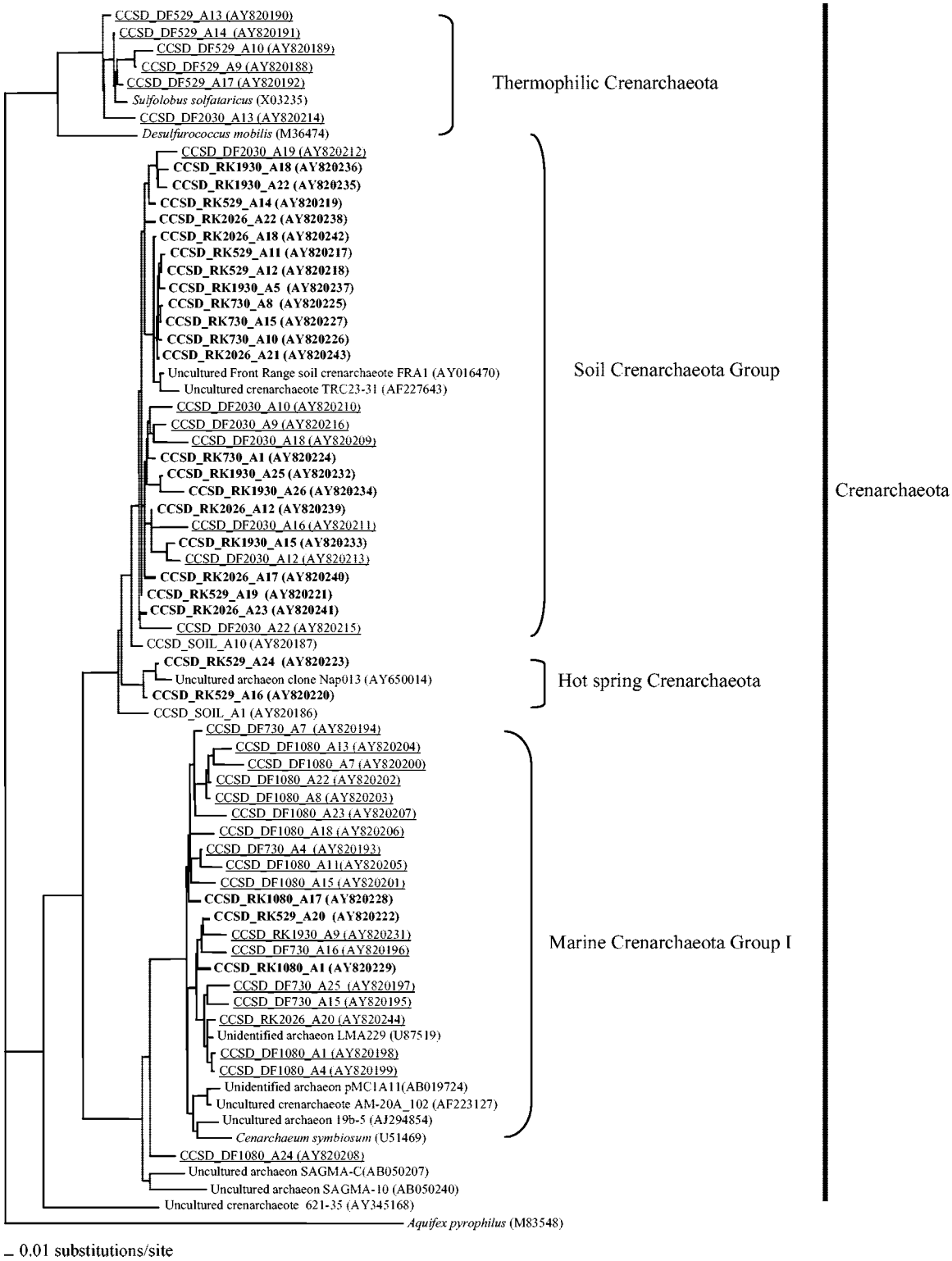



FIG. 7. Phylogenetic relationships of representative phylotypes of archaeal 16S rRNA gene sequences as determined by the neighbor-joining method. Scale bar = 0.01 nucleotide substitution per site.

similar to *S. solfataricus*, a thermophilic archaeon. Except for all 21 sequences from the CCSD_RK1080 sample and six sequences from the CCSD_RK529 sample, all other sequences from the rocks studied formed the second group along with the sequences from the deepest drilling fluid sample, CCSD_DF2030. This group was similar to the soil *Crenarchaeota* group (GenBank description). The third group was closely related to an uncultured archaeon clone, Nap013, obtained

from a hot spring (GenBank description). The fourth group consisted of all 33 sequences from drilling fluid samples CCSD_DF730 and CCSD_DF1080 and nine and four sequences from samples CCSD_RK1080 and CCSD_RK529, respectively. This group was closely related (94 to 98% similarity) to uncultured marine *Crenarchaeota* group I (24).

## DISCUSSION

**Biomass and microbial activity.** The amount of biomass in the UHP rocks was comparable to the amounts observed in similar rock types from other subsurface extreme environments (27) but much lower than the amounts in sediments in various environments (28, 29). This was expected because the UHP rocks had low porosity, low total organic carbon contents, and low levels of other nutrients. Despite these extreme conditions, our culture-based results indicated the presence of active microorganisms [Fe(III) reducers] in the rocks. The acquisition of new isolates from the drilling fluids suggests that novel bacteria were present in the subsurface, and their growth habitats [anaerobic, thermophilic, and alkaliphilic fermenters and Fe(III) reducers] were consistent with the geochemical characteristics of the fluids from depth.

**Microbial diversity.** Microbial life in the deep subsurface has been postulated for a long time, but surface contamination is a critical issue, especially in low-biomass environmental samples (43). The strict quality control used in this study showed that contamination was minimal. Significant changes in bacterial diversity and community structure were associated with environmental gradients in the deep subsurface. Phylogenetic analyses of 16S rRNA gene sequences from the rock samples collected at different depths revealed a significant degree of bacterial diversity, but the archaeal diversity was low. The frequency of 16S rRNA gene clones should be regarded as qualitative information on community composition. Nonetheless, 16S rRNA gene sequence libraries provided valuable descriptions of microbial diversity that allowed comparisons between communities in different environments.

The rocks from all five depths were dominated by bacterial sequences belonging to the *Proteobacteria* lineage, suggesting that this group is one of the most predominant microbial components in terrestrial subsurface. The community structure within the *Proteobacteria* changed with depth, however. Clone sequences belonging to the α-*Proteobacteria* subdivision were present in the shallowest rock (CCSD_RK529), the surface soil, and most of the drilling fluids, but they were absent in the deep rocks (CCSD_RK730 and deeper) and in the deepest drilling fluid sample. Clone sequences belonging to the β-*Proteobacteria* subdivision were predominant in CCSD_RK730 and became less abundant with increasing depth. At the species level, there was a major shift in the community structure with increasing depth. Clone sequences related to aerobic bacteria (e.g., *S. echinoides*) were present only in the shallow rock (CCSD_RK529) and were absent in the deeper samples. Sequences closely related to the metal-reducing bacterium *P. agglomerans* were dominant in the shallow rock samples (CCSD_RK529 and CCSD_RK730) but absent in the deeper samples. Clone sequences belonging to the genus *Pseudomonas* became increasingly abundant with depth. Sequences related to a thermophilic methanotroph, *M. tepidum*, were predominant in the deepest rock sample, and this occurrence was correlated with the high concentration of methane at this depth (0.04%, by weight), as measured by real-time gas chromatography for gases extracted from the circulating drilling fluid.

The bacterial community in the drilling fluid samples was more diverse than that in the rock samples from the same depth and included anaerobic, alkaliphilic, chemoorganotrophic or chemolithoautotrophic, halotolerant or halophilic, nitrate, Fe(III), and sulfate reducers, acetogens, and methanotrophs. This was expected because the drilling fluids contained surface makeup water, clay, mud, and freshly cut rock fragments. In addition, the drilling fluid samples may have contained geological fluids, gases, and formation water from structurally weak shear zones or faults at various depths of the drill hole.

**Correlation between geochemistry and microbiology.** The geochemical conditions and energy sources available tend not only to dictate microbial community composition and species richness but also to constrain the physiological characteristics of the community members. One distinct feature of the clone sequences for the UHP rocks was that most of them were closely related to nitrate-reducing bacteria. This was consistent with the geochemical condition that the rocks had unusually high nitrate concentrations. However, nitrate reducers may not be active, and high concentrations of nitrate remained. Multiple sequences closely related to iron-reducing bacteria were present in the rocks. For the same rock type with the same mineralogy (eclogite; samples CCSD_RK529, CCSD_RK730, CCSD_RK1080, CCSD_RK1930), the proportions of clones related to iron-reducing bacteria (5 of 16, 8 of 28, 0, and 2 of 21 for the CCSD_RK529, CCSD_RK730, CCSD_RK1080, and CCSD_RK1930 samples, respectively [Table 3]) were approximately inversely correlated with the $Fe_2O_3$/FeO ratios of the whole rocks (Table 2), suggesting that iron reducers may have reduced Fe(III) to Fe(II) in the minerals. The presence of Fe(III)-reducing activity in the rock enrichments supported this conclusion. In addition, many clone sequences were related to either alkaliphilic bacteria (such as *A. olivoapovliticus*) or clones previously found in alkaline and saline environments (e.g., Mono Lake in California and the deep sea). Many were resistant to heavy metals, suggesting the existence of saline, alkaline, and possibly metal-rich geochemical environments. In summary, most clone sequences from the UHP rocks were related to microorganisms that have been isolated from deep subsurface environments (such as *Pseudomonas*) or Antarctic lakes. These organisms are generally facultative, heterotrophic, and halotolerant or halophilic and carry out a variety of functions (nitrate, Fe, and sulfate reduction and methanotrophy).

These unique microbiological characteristics suggest that the microbes were present in the UHP rocks in geochemical niches where there were high concentrations of nitrate and ferric iron, high pH values, and possibly saline and cold conditions. We speculate that these microbes were present in various fluid and gas inclusions in minerals such as garnet and pyroxene, where there were nutrients, water, and carbon sources. Because of the low heat conductivity of the rocks and isolated fluid and gas inclusions, fluid and gas inclusions may be out of equilibrium with the in situ subsurface conditions

(such as temperature and pressure), and microbes were expected not to respond to any changes in environmental gradients.

The origins of the fluid and gas inclusions remain to be studied. However, microbial habitats (cold, saline, high nitrate concentration) suggest that at least some fluid and gas inclusions may have been derived from ancient seawater. Microbes might have been trapped inside the fluid and gas inclusions for as long as the age of the inclusions. If this is true, microbes might have survived the subduction and exhumation process. Zheng et al. (44) proposed an ice cream-frying model, in which rapid subduction and exhumation could have preserved localized pockets of fluids that were never heated above the maximal survival temperature for microorganisms when they were subducted. If this is true, the microbes found in this study could have been the same age as the fluid inclusions or older than the fluid inclusions. A recent study (36) showed that if microbes (*Shewanella oneidensis* strain MR-1 and *E. coli* strain MG1655) reside in fluid inclusions, they can maintain physiological and metabolic activity at pressures of 1,200 to 1,600 MPa. They continue to be viable upon transfer to ambient pressure (0.1 MPa). Thus, it is conceivable that the microbes found in this study could have survived the subduction-related UHP (2.8 GPa) metamorphism. However, more research is necessary to test this hypothesis. The type III and IV fluid inclusions in garnet and pyroxene, however, were more likely candidates as sites for microbes as garnet and pyroxene contained high concentrations of P, an essential nutrient for microbial metabolism. In addition, type III and IV fluid inclusions formed at relatively low temperatures during the exhumation of the UHP rocks.

Most of the clones from the rocks were related to mesophilic and psychrophilic microbes and environmental clones, which is inconsistent with the in situ temperature, which ranged from 30 to 60°C. This apparent inconsistency may be reconciled with the ice cream-frying model, in which preservation of cold, localized pockets of fluid inclusions is made possible by rapid subduction and exhumation (44). The presence of mesophilic and psychrophilic microorganisms in these rocks supports this model and argues that these microbes may have come from the ocean floor prior to the subduction process. Oceanic plates were present before the subduction (44), and an oceanic setting is consistent with the psychrophilic habitats of microbes detected in this study (cold environments such as deep-sea sediments or Antarctic lakes).

The psychrophilic nitrate and Fe(III) reducers present in the drilling fluids may have had the same origin as those in the rocks, but the existence of thermophilic bacteria and archaea (such as *S. solfataricus*) in the drilling fluids suggests different origins, and their possible habitats may be pockets of fluids and gases associated with structurally weak shear zones and faults. Because of possible connections to macroscopic flow channels and shear zones, these fluids and gases may be in equilibrium with the modern day geothermal gradient. The presence in sample CCSD_DF529 of *S. solfataricus*, a thermophilic archaeon with an optimal growth temperature of 75°C, is approximately consistent with the in situ temperature at this depth in the borehole.

To the best of our knowledge, this study is the first study to systematically examine the microbial community in UHP rocks. Metamorphic rocks are important constituents of the deep subsurface crust, and our study demonstrated that the biosphere extends into this part of the lithosphere. Metamorphic rocks typically experience high temperatures and pressures, so their porosity and permeability are low. Jenneman et al. (15) observed that bacterial penetration into sandstone with a permeability of <100 mDarcy occurred slowly. The UHP rock samples used in this study had permeability values of around ~0.5 mDarcy. These values were low enough to effectively seal the rocks so that microbial movement was not possible.

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