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Microbial Community in Black Rust Exposed to Hot Ridge Flank Crustal Fluids▽†

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During Integrated Ocean Drilling Program Expedition 301, we obtained a sample of black rust from a circulation obviation retrofit kit (CORK) observatory at a borehole on the eastern flank of Juan de Fuca Ridge. Due to overpressure, the CORK had failed to seal the borehole. Hot fluids from oceanic crust had discharged to the overlying bottom seawater and resulted in the formation of black rust analogous to a hydrothermal chimney deposit. Both culture-dependent and culture-independent analyses indicated that the black-rust-associated community differed from communities reported from other microbial habitats, including hydrothermal vents at seafloor spreading centers, while it shared phylotypes with communities previously detected in crustal fluids from the same borehole. The most frequently retrieved sequences of bacterial and archaeal 16S rRNA genes were related to the genera *Ammonifex* and *Methanothermococcus*, respectively. Most phylotypes, including phylotypes previously detected in crustal fluids, were isolated in pure culture, and their metabolic traits were determined. Quantification of the dissimilatory sulfite reductase (*dsrAB*) genes, together with stable sulfur isotopic and electron microscopic analyses, strongly suggested the prevalence of sulfate reduction, potentially by the *Ammonifex* group of bacteria. Stable carbon isotopic analyses suggested that the bulk of the microbial community was trophically reliant upon photosynthesis-derived organic matter. This report provides important insights into the phylogenetic, physiological, and trophic characteristics of subseafloor microbial ecosystems in warm ridge flank crusts.

The upper 500 m of oceanic crust is porous, which allows active circulation of seawater (7, 14). Fluids passing through ridge flank oceanic crust (1 to 65 million years old) account for 2% of the total volume of seawater (30) and flush this entire volume in about 70,000 years (68). Hence, ridge flank oceanic crust may represent a huge yet mostly ignored microbial habitat that has great significance for the global biogeochemical cycle (5, 6, 12, 17, 20, 23). Since hydrothermal alteration of seawater occurs more gently in ridge flanks than in seafloor spreading centers, ridge flank crustal fluids often contain SO_4^{2-} (66, 69) and even NO_3^- and O_2 (10), which potentially serve as electron acceptors for indigenous microbial communities. Previous studies have revealed the role of microbes in altering cool ridge flank crusts exposed at the seafloor, e.g., via oxidation of S and Fe (12). Additionally, textural and carbon isotopic composition analyses have suggested that microbial alteration of basaltic glass occurs even in high-temperature, deep ridge flank crusts (17, 18). However, very little is known about the diversity, physiology, and activity of microorganisms within the warm ridge flank crusts, in part due to the difficulty of recovering contamination-free samples from deep crust.

Recently, circulation obviation retrofit kits (CORKs) have provided unprecedented opportunities for microbiological investigation of oceanic ridge flanks (6, 7, 23, 35). To date, two microbial diversity studies have been performed using hot crustal fluids collected from a CORK deployed on the eastern flank of Juan de Fuca Ridge (6, 23). The presence of phylogenetically diverse *Archaea* and *Bacteria*, potentially transported from ridge flank crust, was revealed by culture-independent methods (6, 23). In addition, the chemical composition of CORK fluids suggested that various microbial processes occur

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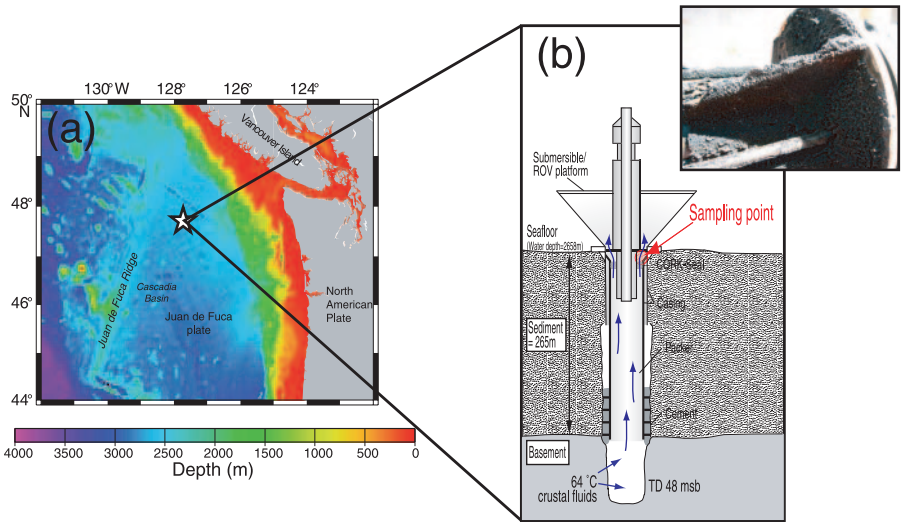


FIG. 1. (a) Location of CORK 1026B on the eastern flank of the Juan de Fuca Ridge. (b) Schematic diagram of CORK 1026B and borehole (modified from reference 16). The inset shows the black rust used in this study. msb, meters subbasement.

within ridge flanks, including thermophilic sulfate reduction, ammonification of nitrate, and fermentation (6, 69). However, it remains to be determined which microorganisms are responsible for which microbial processes, and the chemical signatures might also be explained by abiotic hydrothermal alteration of seawater and diffusive exchange with the overlying sediment pore waters (6, 67, 69). In addition, the energy sources for the potential microbial activities remain unknown (6). In 2004, we retrieved an old CORK in order to replace it with a new one to prevent further leakage of crustal fluids (16). Visual inspection onboard revealed that there was a black rust deposit on the old CORK (16). As a further step toward understanding the microbial ecosystem within ridge flank crusts, we investigated the microbial community in the black rust by using both culture-dependent and culture-independent methods.

MATERIALS AND METHODS

Study site and sample collection. The old CORK was installed in borehole B at Ocean Drilling Program Site 1026 on the eastern flank of the Juan de Fuca Ridge during Ocean Drilling Program Leg 168 in July 1996 (15) (Fig. 1) (47°46′N, 127°46′W; 3.5-million-year-old crust; approximately 100 km from the ridge axis; water depth, 2,658 m; total penetration, 295 m; basement penetration, 48 m). CORKs consist of two parts: (i) a data logger and fluid sampling port sitting on the seafloor and (ii) instruments installed in the sealed borehole below (for a review, see reference 2). When the borehole was drilled for the deployment of CORK 1026B, 64°C crustal fluids flowed up at a rate of 84 liters min^{-1} (69). CORK 1026B did not completely seal the borehole, which allowed hot fluids to seep out at a rate of 3.1 liters min^{-1} (7). When we recovered this CORK for replacement on 4 August 2004, we found black rust on the CORK steel surface, immediately above the seal (Fig. 1). The black rust (30 cm^3) was scaled and slurried for cultivation with sterile synthetic seawater (50 ml) containing 0.05% (wt/vol) $Na_2S \cdot 9H_2O$ under an N_2 atmosphere (40). Approximately 100 cm^3 of the sample was stored at −80°C for other analyses. For optical microscopic observation, 5 cm^3 of the sample was fixed with filter-sterilized synthetic seawater (30 ml) containing 3.7% (wt/vol) formaldehyde. A total cell count (cells per cm^3) was obtained onboard ship by direct cell counting of the formaldehyde-fixed slurry with DAPI (4′,6-diamidino-2-phenylindole) using epifluorescence microscopy (47).

Mineralogical analysis. The elemental composition and mineral components of the black rust were determined with a scanning electron microscope or with a microprobe using energy-dispersive X-ray spectroscopy (EDX) (model INCA Energy 200; Oxford Instruments, Oxford, United Kingdom), powder X-ray diffraction pattern analysis (model Rint 2000; Rigaku Corporation, Tokyo, Japan), and selected area electron diffraction (SAED) pattern analysis as previously described (54, 55).

Cultivation test. To estimate the abundance and diversity of culturable microorganisms, a three-tube most-probable-number (MPN) test using a total of 32 different cultivation conditions (see Table S1 in the supplemental material) was performed onboard as previously described (41, 42). The cultivation conditions that gave positive enrichments are shown in Table 1. Pure cultures were obtained from the highest positive dilution tube by using the dilution-to-extinction technique (1). Sulfate-reducing strains and methanogens were identified by checking production of H_2S and by checking autofluorescence by UV microscopy, respectively. The purity was routinely checked by microscopy.

Phylogenetic analysis of pure cultures. Phylogenetic analysis of pure cultures was performed as described previously (41, 42). Genomic DNA was extracted from cell pellets of each isolate with a Soil DNA Mini prep kit (MO BIO Laboratories, Inc., Solana Beach, CA). The 16S rRNA gene was amplified by PCR with primers Eubac27F and 1492R (34) for the bacterial rRNA gene or with primers Arch21F (8) and 1492R (34) for the archaeal rRNA gene. Nearly complete sequences of the PCR products were directly determined for both strands. Sequences were used for similarity analysis with databases by using the FASTA algorithm of the DNA Data Bank of Japan (DDBJ) (36).

16S rRNA gene clone library. Microbial DNA was directly extracted from the microbial community associated with the black rust sample using a Soil DNA Mega prep kit (MO BIO Laboratories). As a negative control to check for experimental contamination, a blank tube was subjected to the same process. The primers described above were used. The PCR conditions used have been described previously (29). The presence of members of the *Thermococcaceae* was also assessed by PCR using a *Thermococcaceae*-specific primer set consisting of primers TcPc173F and TcPc589R (52). For a positive control, DNA extracted from a chimney structure in the Iheya North hydrothermal field (41) was used as a template. Each amplicon was purified, and cloning and sequencing were performed by a previously described procedure (29). Primer Eubac27F or Arch21F was used for partial sequencing (approximately 500 bp) of the insert to determine the phylogenetic clone type (phylotype). Clones with ≥97% sequence similarity were assigned to the same phylotype. Approximately 1,400 bp of each representative rRNA gene clone sequence was determined for both strands. Chimeric sequences were searched by checking secondary structure anomalies, using the Bellerophon program (25) and fractional treeing (37). None of the sequences were found to be chimeric.

Construction of phylogenetic tree. To determine phylogenetic affiliations of isolates and environmental sequences, 16S rRNA gene sequences were compiled by using ARB, version 20030822 (38), and were aligned with a database (26) updated with sequences from the DDBJ. The resulting alignments were manually checked against known secondary structure regions. Phylogenetic analyses were restricted to nucleotide positions that could be unambiguously aligned. Phylo-

TABLE 1. Culturable population and phylogenetic characteristics of isolates from the black rust deposit on CORK 1026B

Strain	MPN (cells per cm^3)	Temp (°C)	Medium (reference)	Gas phase	Closest relative (% similarity)[a]
Methanogens					
Mc70	1.3×10^3	70	MMJ (61)	80% H_2–20% CO_2 (350 kPa)	*Methanothermococcus thermolithotrophicus* (99.5)
Mc55	1.3×10^3	55	MMJ (61)	80% H_2–20% CO_2 (350 kPa)	*Methanothermococcus thermolithotrophicus* (99.3)
Mc37	5.9×10^2	37	MMJ (61)	80% H_2–20% CO_2 (350 kPa)	*Methanothermococcus thermolithotrophicus* (99.1)
Ep55	5.9	55	MMJHS (61)	80% H_2–20% CO_2 (350 kPa)	*Methanothermococcus thermolithotrophicus* (99.5)
Ep70	5.9	70	MMJHS (61)	80% H_2–20% CO_2 (350 kPa)	*Methanothermococcus thermolithotrophicus* (99.4)
Sulfate reducers					
Tc37	49.6	37	MJYPGS (41)	100% N_2 (200 kPa)	*Desulfuromonas michiganensis* (92.2)
Spi55	5.0	55	MEtSR (this study)[b]	80% N_2–20% CO_2 (250 kPa)	*Desulfonatronovibrio hydrogenovorans* (91.4)
Srb55	4.9	55	MEtSR2 (this study)[c]	80% H_2–20% CO_2 (350 kPa)	*Desulfotomaculum geothermicum* (96.1)
PM70-1	ND[h]	70	DSMZ63-2 (this study)[d]	80% H_2–20% CO_2 (200 kPa)	*Archaeoglobus veneficus* (95.1)
Fermenters					
Ag70	49.6	70	MJYS (41)	80% H_2–20% CO_2 (350 kPa)	*Thermosipho melanesiensis* (86.4)
Ag55	4.9	55	MJYS (41)	80% H_2–20% CO_2 (350 kPa)	*Thermosipho melanesiensis* (87.1)
Ag-C55	5.9	55	MJYS2 (this study)[e]	100% H_2 (350 kPa)	*Halothermothrix orenii* (92.7)
Tc55	5.9	55	MJYPGS (41)	100% N_2 (200 kPa)	*Bacillus* sp. strain BR (98.6)
Bal55	2.0	55	MEtSR (this study)	80% N_2–20% CO_2 (250 kPa)	*Desulfotomaculum salinum* (91.5)
Kimo37	2.0	37	MJYP (50)	100% N_2 (200 kPa)	*Cytophaga* sp. strain AN-BI4 (88.6)
Yos55	2.0	55	MJFM (this study)[f]	80% N_2–20% CO_2 (250 kPa)	*Thermohalobacter berrensis* (91.5)
TH70-3	ND	70	MSTH (this study)[g]	100% N_2 (200 kPa)	*Thermosipho atlanticus* (98.1)

[a] Determined by using the FASTA program of the DDBJ.
[b] MEtSR contained 10 mM ethanol, 0.1 g yeast extract, 1 g $NaHCO_3$, 1.5 g Na_2SO_4, 0.5 g Na_2S, and 1 mg resazurin in 1 liter of MJ synthetic seawater (49).
[c] MEtSR2 contained 0.5 g acetate, 0.5 g lactate, 0.5 g pyruvate, and 0.5 g, citrate instead of ethanol in 1 liter of MEtSR medium.
[d] DSMZ63-2 was DSMZ medium 63 containing 2.5% $Fe(NH_4)_2(SO_4)_2$ instead of $FeSO_4$.
[e] MJYS2 was MJYS medium containing no $NaHCO_3$.
[f] MJFM contained 0.5 g yeast extract, 0.5 g peptone, 0.5 g formate, 0.5 g lactate, 0.5 g acetate, 0.5 g glucose, 0.5 g maltose, and 0.5 g citrate in 1 liter of MJ synthetic seawater (49).
[g] MSTH contained 1 g yeast extract, 1 g peptone, 1 g $NaHCO_3$, 0.5 g Na_2S, and 1 mg resazurin in 1 liter of marine medium (70) amended with SL-10 trace mineral solution (71).
[h] ND, not determined.

genetic trees were generated by a distance method using PAUP* 4.0b10 (57) and ARB. Distances were estimated with the Jukes-Cantor correction. Bootstrap analyses with 100 trial replications were used to obtain confidence estimates for the tree topologies.

Dissimilatory sulfite reductase (*dsrAB*) gene clone library. Amplification, purification, cloning, and sequencing of the *dsrAB* genes were performed as previously described (44) with DNA obtained as described above. Primers DSR1Fdeg and DSR4Rdeg (32) were used. *dsrAB* gene clones with ≥95% sequence similarity were assigned to the same clone type. Likewise, the *dsrAB* genes were also amplified from the sulfate-reducing strains obtained in this study. Approximately 2,000 bp of the *dsrAB* gene sequence of each representative clone and sulfate-reducing strain were determined for both strands by using internal primers as described by Nakagawa et al. (44). Deduced amino acid sequences were aligned with prokaryotic dissimilatory sulfite reductase amino acid sequences obtained from DDBJ using the CLUSTAL X software (63). A neighbor-joining tree based on Kimura two-parameter distances was constructed by using CLUSTAL X. A bootstrap analysis with 100 trial replications was performed.

Quantification of archaeal 16S rRNA genes. Quantification of archaeal 16S rRNA genes in the whole microbial 16S rRNA gene assemblage was performed by real-time PCR with TaqMan probes as previously described (58). A dilution series of a DNA sample was prepared and assayed with a universal 16S rRNA gene mixture and an archaeal 16S rRNA gene mixture (58) as standards for quantification of the whole microbial 16S rRNA gene and the archaeal 16S rRNA gene, respectively.

Quantification of *dsrAB* genes. The abundance of *dsrAB* genes in the sample was estimated by real-time PCR with a SYBR Premix Ex *Taq* kit (TaKaRa, Shiga, Japan) using primers DSR1Fdeg (32) and int350R (5′-GTGCAGCTCG TCCTGGTA-3′). The int350R primer was designed to anneal to the conserved region of the entire *dsrAB* genes sequenced in this study. PCR-amplified *dsrAB* genes from the sample were quantified spectrometrically and used as standards. Dilution series of the DNA sample and standards were prepared and assayed by using a real-time PCR system (model 7500; Applied Biosystems, Foster City, CA). The thermal cycle was as follows: initial denaturation of the template DNA at 95°C for 60 s, followed by 40 cycles of amplification in which each cycle consisted of denaturation at 95°C for 5 s, primer annealing, and extension at 60°C for 34 s. Following amplification, a melting curve analysis of the amplicon was performed. Melting temperature analysis confirmed that the specificity of detection was high in all cases.

Bulk sulfur isotopic analysis. Prior to analysis, the ground sample was washed with distilled water three times and dried. Tin capsules containing a reference (IA-R036, IAEA-S-1, and IA-R025; Iso-Analytical, Cheshire, United Kingdom) or a washed sample plus vanadium pentoxide catalyst were loaded into the automatic sampler of an elemental-analysis isotope ratio mass spectrometer (ANCA-GSL; SerCon, Cheshire, United Kingdom). The capsules were combusted in the presence of oxygen at 1,080°C. The tin capsules flash combusted, raising the temperature in the region of the sample to ~1,700°C. Sulfur dioxide was separated from N_2 and CO_2 using a packed gas chromatography (GC) column at 45°C. The resulting SO_2 peak entered the ion source of the isotope ratio mass spectrometer, where it was ionized and accelerated. The measured isotopic composition was expressed as $\delta^{34}S_{VCDT}$, which was defined as follows: $\delta^{34}S_{VCDT} = [(^{34}S/^{32}S)_{sample}/(^{34}S/^{32}S)_{standard} - 1] \times 10^3$, where $(^{34}S/^{32}S)_{standard}$ is the abundance ratio for the Canyon Diablo troilite (CDT).

Bulk carbon isotopic analysis. Bulk carbon isotopic analysis was performed as described previously (56). Ground, lyophilized, and acid-fumed samples were analyzed by using a Thermo Electron DELTA^{plus} Advantage mass spectrometer connected to an elemental analyzer (EA1112) through a ConFlo III interface. The measured isotopic composition was expressed as $\delta^{13}C$, which was defined as follows: $\delta^{13}C_{VPDB} = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1] \times 10^3$, where $(^{13}C/^{12}C)_{standard}$ is the abundance ratio for the Pee Dee belemnite (PDB).

Compound-specific carbon isotopic analysis. Total lipid was extracted from a lyophilized ground sample using a single-phase chloroform-methanol buffer system of Bligh and Dyer (3). Fatty acid methyl esters (FAMEs) were prepared with anhydrous methanol HCl at 100°C for 3 h (33). They were then analyzed by using a GCQ gas chromatography-mass spectrometry system (Shimadzu, Kyoto, Japan) and GC-carbon isotope ratio mass spectrometry with a Thermo Electron DELTA^{plus} Advantage mass spectrometer connected to a GC (Agilent 6890; Agilent, Mountain View, CA) through a GC/C/C/III interface (56). Standard nomenclature was used for fatty acids, which were designated as follows: $C_{X:Y}$, where X is the number of carbon atoms and Y is the number of double bonds. The isotopic compositions of the FAMEs were determined with an internal

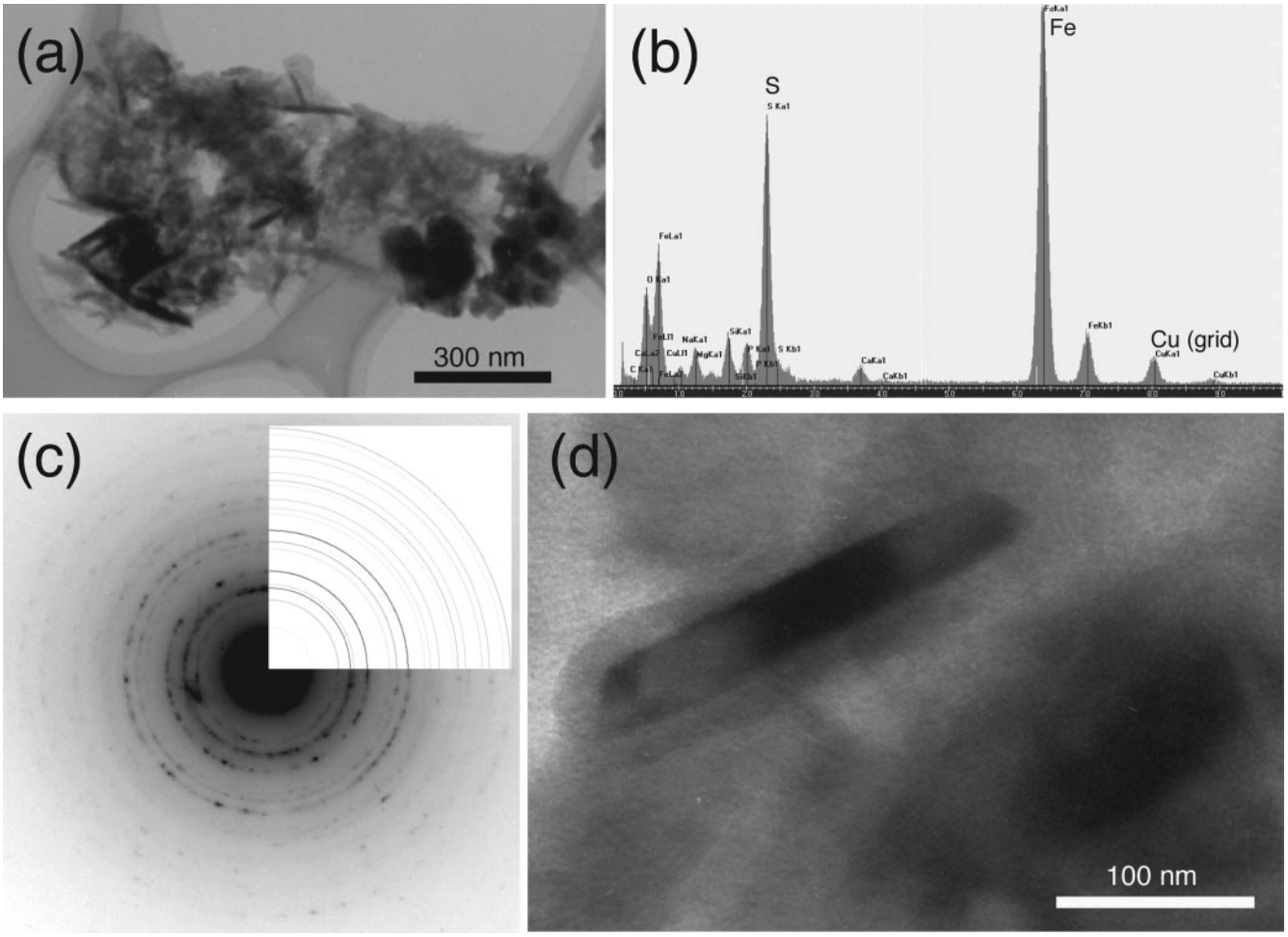


FIG. 2. (a and b) Transmission electron microscopy image (a) and EDX spectrum (b) of a prokaryotic cell in the black rust that had accumulated iron and sulfur. (c and d) SAED pattern (c), showing that a crystal accumulated on the cell surface (d) is greigite.

isotopic standard (for $C_{19:0}$, $\delta^{13}C_{VPDB} = -29.80‰$) with correction for the additional carbon atom from methanol-derivatizing reagents ($\delta^{13}C_{VPDB} = -39.04‰$).

Nucleotide sequence accession numbers. Sequences obtained in this study have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the following accession numbers: AB260037 to AB260053 for 16S rRNA genes of isolates, AB260054 to AB260068 for representative 16S rRNA gene clones, AB260069 to AB260071 for *dsrAB* genes of isolates, and AB260072 to AB260076 for representative *dsrAB* gene clones.

RESULTS

Sample description. We collected the black rust from a borehole seal instrument, CORK 1026B, which had been deployed for approximately 8 years on a 295-m-deep borehole in the eastern flank of Juan de Fuca Ridge (Fig. 1). CORK 1026B, like a deep-sea hydrothermal vent, began leaking hot crustal fluids soon after its deployment (7). The temperature of the effluent fluids at the time of recovery was not determined, but previous measurements at the seafloor indicated that it was approximately 54°C in 1997 to 1999 (6) and 62°C in 2002 (23). The black rust was found immediately above the CORK seal, where the leaking hot crustal fluids and cold bottom seawater mixed (Fig. 1). The high temperature surrounding the microbial habitat was clearly confirmed by the dominance of thermophiles (described below). The total cell density was estimated to be 1.9×10^6 cells per cm^3.

Mineralogical analyses. The black rust was attracted to a magnet, indicating that the sample contained magnetic iron-bearing minerals. Scanning electron microscope-EDX analysis revealed that the bulk of the sample consisted of O (65.5 atom%), Si (9.0 atom%), Al (8.2 atom%), C (5.2 atom%), Fe (4.5 atom%), Na (1.8 atom%), Mg (1.8 atom%), S (1.3 atom%), and Cl (1.2 atom%) as the primary (>1 atom%) elements. When the sample was observed with transmission electron microscopy, some cells that contained fine crystals (length, 30 to 350 nm) were found (Fig. 2a and d). EDX and SAED pattern analyses revealed that the crystals were greigite (Fe_3S_4) (Fig. 2b and c). It is known that sulfate-reducing bacteria form extracellular iron sulfide minerals, such as greigite, during dissimilatory sulfate reduction (48). The formation of greigite crystals thus suggested that there was microbial sulfate reduction in the CORK habitat.

Cultivation test. Microorganisms, including both *Archaea* and *Bacteria*, were quantitatively cultured and isolated in pure cultures. The overall size of the culturable population was

approximately 1.3×10^3 cells per cm^3. The numerically most abundant culturable population consisted of thermophilic and hydrogenotrophic methanogens (Table 1). Although the MPNs for other isolates were small, a variety of fermenters and sulfate reducers were also cultured (Table 1). Based on the 16S rRNA gene sequences, most of these isolates were only distantly related to previously cultured microorganisms and represented novel species or genera (Table 1). Among the isolates, thermophilic fermenters belonging to the order *Thermotogales* (i.e., strains Ag55 and Ag70) exhibited 99.3 and 99.1% 16S rRNA gene similarity with an environmental clone previously detected in fluids from the same borehole (6) (Fig. 3a). Likewise, sulfate-reducing strains Spi55 (a member of the *Desulfovibrionales*), Srb55 (*Desulfotomaculum* sp.), and PM70-1 (*Archaeoglobus* sp.) and fermenting strains Bal55 (a member of the *Clostridiales*) and TH70-3 (*Thermospipho* sp.) were closely related to other CORK fluid clones (6, 23) (Fig. 3a and b). None of the enrichments incubated under aerobic, microaerobic, or nitrate-reducing conditions were successful. It should be noted that none of the microorganisms commonly cultured from various deep-sea hydrothermal vent environments (i.e., members of the *Thermococcales*, *Aquificales*, and epsilon-*Proteobacteria* [40]) were cultured, even though the cultivation conditions employed in this study have been successfully used for these microorganisms (41, 42, 43, 59, 60). These results indicated that the black-rust-associated community differed from communities reported for deep-sea hydrothermal vents at seafloor spreading centers, while it shared phylotypes with communities previously detected in crustal fluids from the same borehole (6, 23). All MPNs were small compared to the total cell count. This may have resulted in part from the fact that the recovered CORK was exposed to oxic seawater and even to air for several hours due to the complex ship operation (16).

Culture-independent molecular analyses. DNA was successfully extracted from black rust and used to construct libraries of the 16S rRNA genes of *Bacteria* and *Archaea* and the *dsrAB* genes. Negative controls yielded no amplification in any of the PCRs.

(i) Diversity of bacterial 16S rRNA gene. Ten different phylotypes were identified from the bacterial 16S rRNA gene library (Fig. 3a). None of these phylotypes have been detected in any natural deep-sea vent environment. As observed for the microbial community in CORK fluid (6), clones most closely related to *Ammonifex degensii* were detected most frequently (clonal frequency, 86%) (Fig. 3a). Based on multiple alignments with more than 13,000 different rRNA gene sequences in our database, we found that some of the *Ammonifex* group clones sequenced in this study had characteristic long inserts (up to 93 bp long) between *Escherichia coli* positions 462 and 469. *A. degensii* $KC4^T$ is an extremely thermophilic, facultatively chemolithoautotrophic, low-G+C-content, gram-positive bacterium that was isolated from a terrestrial hot spring and grows via hydrogen, formate, or pyruvate oxidation coupled with nitrate, sulfate, or S^0 reduction or pyruvate fermentation (24). Nevertheless, since the levels of sequence similarity between the *Ammonifex* group clones and *A. degensii* $KC4^T$ were less than 91% (Fig. 3a), the dominant *Ammonifex* group bacteria may have different physiological characteristics than *A. degensii* $KC4^T$. The relatively minor bacterial sequences were also affiliated with other subgroups in the low-G+C-content gram-positive group (Fig. 3a). The close relatives of the less frequently detected bacteria (e.g., *Carboxydocella thermautotrophica* [53]) have been found in terrestrial thermal habitats but not in deep-sea environments.

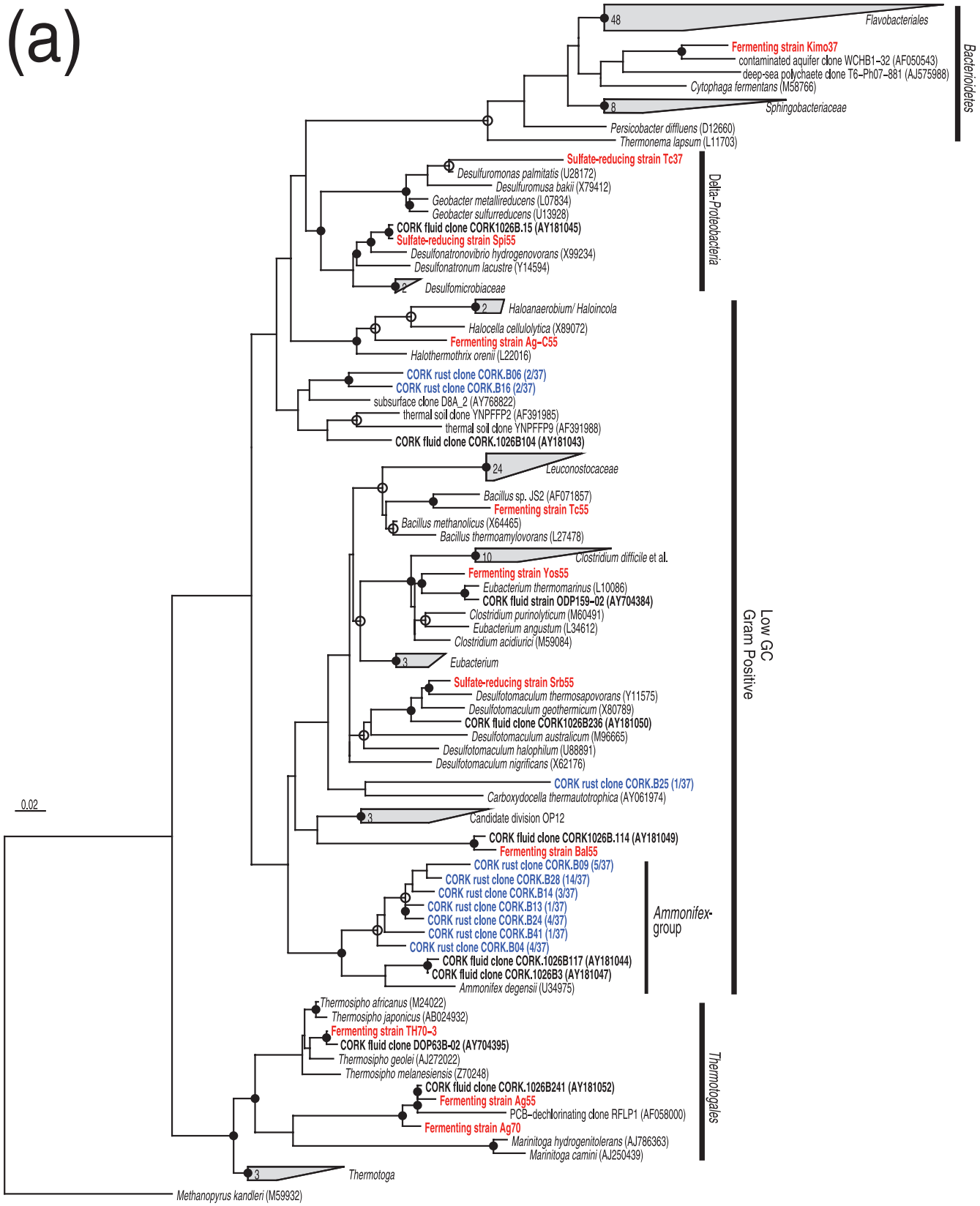
(ii) Diversity and quantification of archaeal 16S rRNA gene. Five different archaeal phylotypes were identified (Fig. 3b). The 16S rRNA gene sequence of the most frequently detected archaeal phylotype (clonal frequency, 82%) was 99.8 to 99.9% similar to the sequences of thermophilic, hydrogenotrophic methanogens isolated in this study (Fig. 3b). No methanogens were detected in previous CORK fluid surveys (6, 23). The difference in community structure might be related to the fact that we sampled a solid formation, whereas in previous studies fluids were sampled. Archaeal sequences affiliated with ANME-2b, marine benthic group E, and the miscellaneous crenarchaeotic group, all of which are dominant archaeal groups in other deep-sea environments, such as methane seeps (45), pelagic sediments (28, 29, 65), and hydrothermal sediments (62), were detected less frequently (Fig. 3b). The absence of detectable *Thermococcaceae* members, hyperthermophilic archaea commonly found in natural deep-sea hydrothermal vent environments, was confirmed by PCR using *Thermococcaceae*-specific primers (52) (see Fig. S1 in the supplemental material).

Whereas the methanogenic archaea represented the most abundant culturable population, the total archaeal 16S rRNA gene population represented only 0.04% (standard deviation, 0.017%; $n = 3$) of total microbial 16S rRNA gene assemblage. These results indicated that members of the *Bacteria* with low culturability were numerically dominant and members of the *Archaea* with high culturability were a minority.

(iii) Diversity and quantification of *dsrAB* genes. Five different groups were identified from the *dsrAB* gene library. The *dsrAB* gene clones formed a new clade, which was most closely related to a *Desulfotomaculum* species (Fig. 4). This clade was distantly related to *dsrAB* genes of sulfate-reducing strains isolated in this study (Fig. 4).

The abundance of *dsrAB* genes was estimated to be 9.5×10^7 copies per cm^3 (standard deviation, 2.5×10^7 copies per cm^3; $n = 3$). This abundance is approximately 10 times higher than the total cell count estimated by DAPI staining. Assuming that all cells of sulfate reducers have a single copy of the *dsrAB* genes, as suggested by the genome sequences of a variety of sulfate-reducing bacteria, the total cell count was most probably underestimated, potentially due to the dominance of cells accumulating greigite (Fig. 2) or spore-forming bacteria impermeable to DAPI. Considering the microbial rRNA gene community structure, the abundantly detected *dsrAB* genes were potentially derived from the *Ammonifex* group bacteria. This hypothesis is consistent with the ability of *A. degensii* $KC4^T$ to grow via dissimilatory reduction of sulfate (24).

Stable isotopic analysis. The $\delta^{34}S_{VCDT}$ value of the bulk sample was determined to be 5.1‰ (standard deviation, 0.01‰; $n = 2$), which is depleted in ^{34}S compared to sulfate in seawater (21‰) (51). As the sulfide concentration in the discharged fluids of the borehole was extremely low (<0.0005 mmol per kg) (6), the depletion of ^{34}S probably resulted from microbial sulfate reduction in situ. This conclusion is sup-


(a)
48
Flavobacteriales
Fermenting strain Kimo37
contaminated aquifer clone WCHB1-32 (AF050543)
deep-sea polychaete clone T6-Ph07-881 (AJ575988)
Cytophaga fermentans (M58766)
8
Sphingobacteriaceae
Bacteriodetes
Persicobacter diffluens (D12660)
Thermonema lapsum (L11703)
Sulfate-reducing strain Tc37
Desulfuromonas palmitatis (U28172)
Desulfuromusa bakii (X79412)
Geobacter metallireducens (L07834)
Geobacter sulfurreducens (U13928)
CORK fluid clone CORK1026B.15 (AY181045)
Sulfate-reducing strain Spi55
Desulfonatronovibrio hydrogenovorans (X99234)
Desulfonatronum lacustre (Y14594)
2
Desulfomicrobiaceae
Delta-Proteobacteria
2
Haloanaerobium/ Haloincola
Halocella cellulolytica (X89072)
Fermenting strain Ag-C55
Halothermothrix orenii (L22016)
CORK rust clone CORK.B06 (2/37)
CORK rust clone CORK.B16 (2/37)
subsurface clone D8A_2 (AY768822)
thermal soil clone YNPFFP2 (AF391985)
thermal soil clone YNPFFP9 (AF391988)
CORK fluid clone CORK.1026B104 (AY181043)
24
Leuconostocaceae
Bacillus sp. JS2 (AF071857)
Fermenting strain Tc55
Bacillus methanolicus (X64465)
Bacillus thermoamylovorans (L27478)
10
Clostridium difficile et al.
Fermenting strain Yos55
Eubacterium thermomarinus (L10086)
CORK fluid strain ODP159-02 (AY704384)
Clostridium purinolyticum (M60491)
Eubacterium angustum (L34612)
Clostridium acidiurici (M59084)
3
Eubacterium
Sulfate-reducing strain Srb55
Desulfotomaculum thermosapovorans (Y11575)
Desulfotomaculum geothermicum (X80789)
CORK fluid clone CORK1026B236 (AY181050)
Desulfotomaculum australicum (M96665)
Desulfotomaculum halophilum (U88891)
Desulfotomaculum nigrificans (X62176)
CORK rust clone CORK.B25 (1/37)
Carboxydocella thermautotrophica (AY061974)
3
Candidate division OP12
CORK fluid clone CORK1026B.114 (AY181049)
Fermenting strain Bal55
CORK rust clone CORK.B09 (5/37)
CORK rust clone CORK.B28 (14/37)
CORK rust clone CORK.B14 (3/37)
CORK rust clone CORK.B13 (1/37)
CORK rust clone CORK.B24 (4/37)
CORK rust clone CORK.B41 (1/37)
CORK rust clone CORK.B04 (4/37)
CORK fluid clone CORK.1026B117 (AY181044)
CORK fluid clone CORK.1026B3 (AY181047)
Ammonifex degensii (U34975)
Ammonifex-group
Low GC Gram Positive
0.02
Thermosipho africanus (M24022)
Thermosipho japonicus (AB024932)
Fermenting strain TH70-3
CORK fluid clone DOP63B-02 (AY704395)
Thermosipho geolei (AJ272022)
Thermosipho melanesiensis (Z70248)
CORK fluid clone CORK.1026B241 (AY181052)
Fermenting strain Ag55
PCB-dechlorinating clone RFLP1 (AF058000)
Fermenting strain Ag70
Marinitoga hydrogenitolerans (AJ786363)
Marinitoga camini (AJ250439)
Thermotogales
3
Thermotoga
Methanopyrus kandleri (M59932)

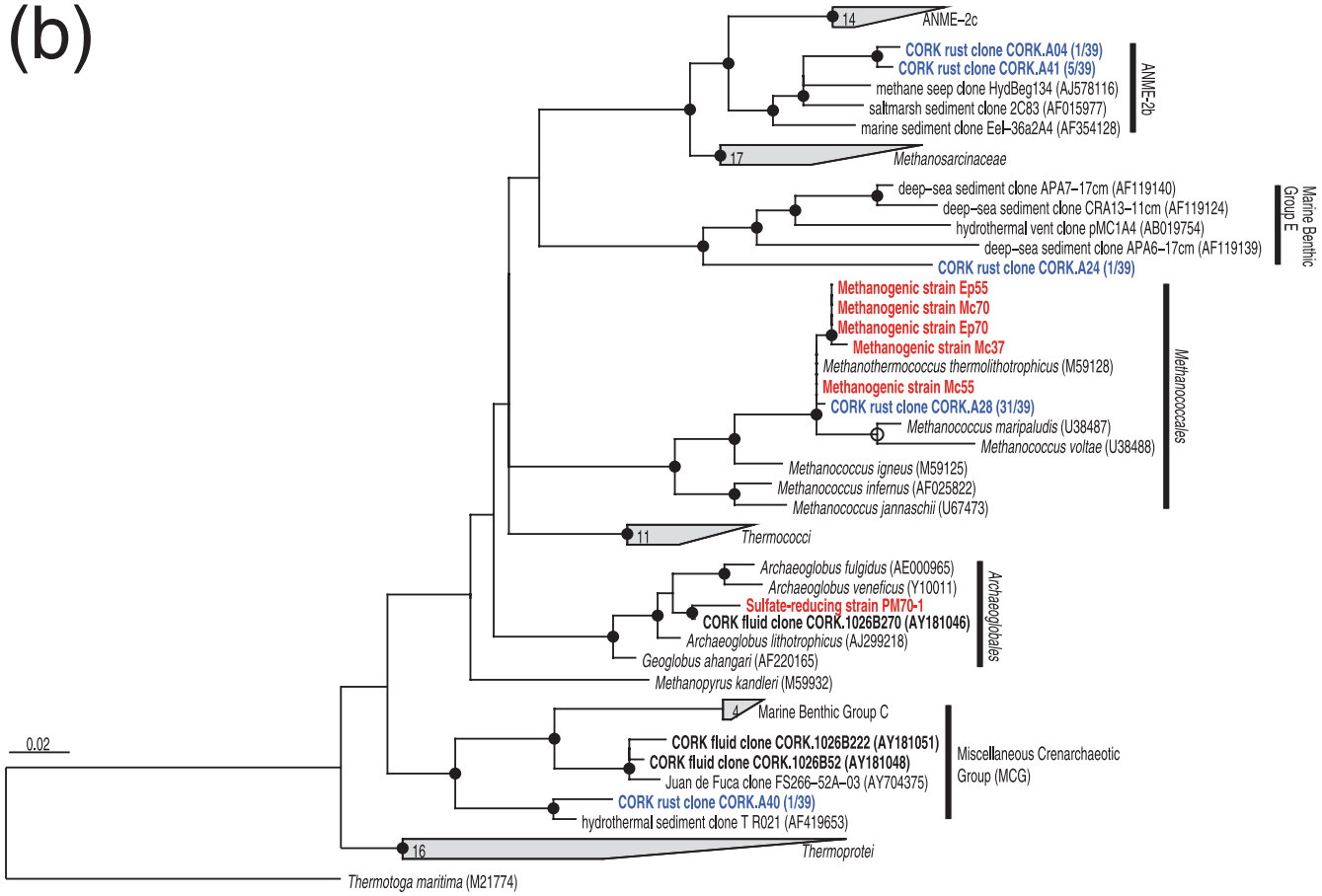


FIG. 3. Phylogenetic relationships of isolates and representative environmental clones as determined by neighbor-joining analysis of 16S rRNA gene sequences. Trees were constructed by using 419 (a) and 411 (b) sites that could be unambiguously aligned. Some sequences are indicated as follows: red, isolates obtained in this study; blue, clones sequenced in this study; boldface black, clones and isolates obtained in previous fluid surveys (6, 23). The remaining sequences were obtained from DDBJ. The clonal frequency of each representative clone obtained in this study and DDBJ accession numbers are shown in parentheses. Branch points conserved with bootstrap values of >75% (solid circles) and with bootstrap values of 50 to 74% (open circles) are indicated. Some groups are represented by shaded trapezoids that indicate the numbers of sequences. Scale bar = 0.02 expected change per nucleotide position. (a) Tree indicating the phylogenetic relationship among members of the *Bacteria*. (b) Tree indicating the phylogenetic relationship among members of the *Archaea*. PCB, polychlorinated biphenyl.

ported by the detection of abundant *dsrAB* genes and the formation extracellular greigite, as observed by electron microscopy (Fig. 2).

The $\delta^{13}C_{VPDB}$ value of total organic carbon in the sample was −22.1‰. The carbon isotopic compositions of FAMEs from the sample were as follows: for $C_{16:1}$, 2.7 area% and $\delta^{13}C_{VPDB}$ value of −45.4‰ ± 1.7‰ (mean ± standard deviation; $n = 3$); for $C_{16:0}$, 46.4 area% and $\delta^{13}C_{VPDB}$ value of −27.4‰ ± 0.6‰ (mean ± standard deviation; $n = 3$); for $C_{17:0}$, 10.6 area% and $\delta^{13}C_{VPDB}$ value of −44.1‰ ± 0.5‰ (mean ± standard deviation; $n = 3$); for $C_{18:1}$, 14.1 area% and $\delta^{13}C_{VPDB}$ value of −24.4‰ ± 1.0‰ (mean ± standard deviation; $n = 3$); and for $C_{18:0}$, 26.2 area% and $\delta^{13}C_{VPDB}$ value of −24.9‰ ± 1.3‰ (mean ± standard deviation; $n = 3$). Of the FAMEs, $C_{16:1}$ and $C_{17:0}$ were minor but significantly depleted in ^{13}C relative to other FAMEs, indicating that there were at least two carbon sources or two types of carbon metabolism in the microbial community.

DISCUSSION

We characterized the microbial community inhabiting a black rust deposit on a CORK borehole observatory on the eastern flank of Juan de Fuca Ridge. Although the black rust had been exposed to hot ridge flank crustal fluids, this habitat cannot be considered a natural microbial habitat for obvious reasons. Hence, it is unlikely that the microbial community in ridge flank crust is identical to the community characterized in this study. However, many relatives of microorganisms found in black rust were previously detected in crustal fluids discharged from the CORK but not in bottom seawater or sediments (6, 23). It appears very likely that the microorganisms were transported and supported by the hot ridge flank crustal fluids, although the crustal fluids might be affected by corrosion of casing and CORK steel (6, 23, 69). Efforts have been made to develop technologies for collecting contamination-free ridge flank crustal fluids (2). This study is the first study in which potentially dominant and indigenous inhabitants of

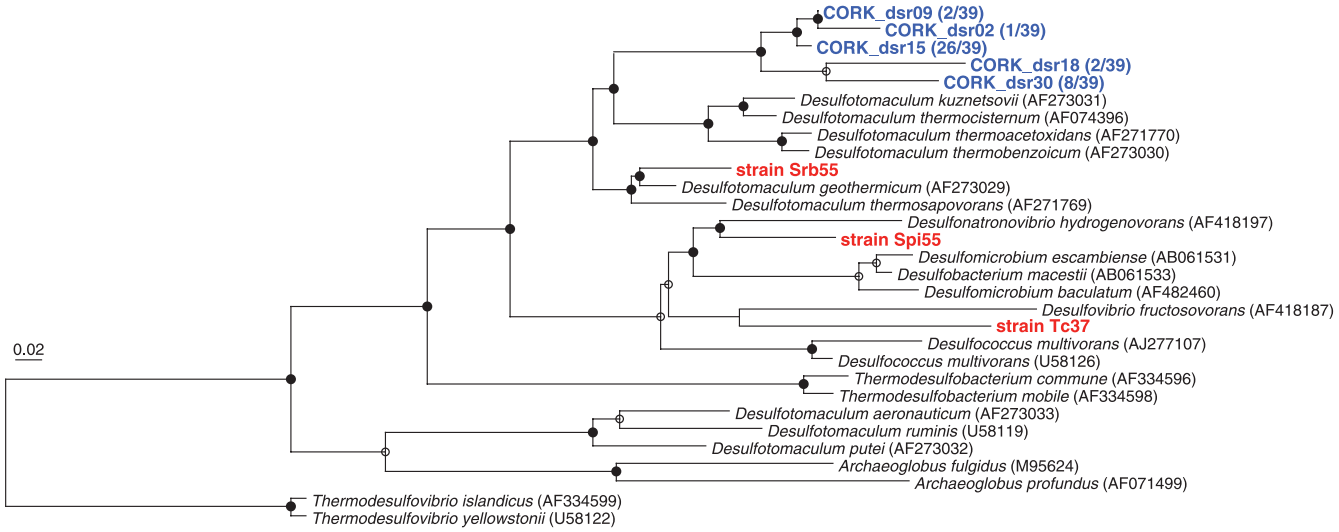


FIG. 4. Phylogenetic tree based on the deduced amino acid sequences of the *dsrAB* genes of representative clones and sulfate-reducing strains. The tree was constructed by using 356 amino acid sequences. See the legend to Fig. 3 for additional information.

warm ridge flank crust were isolated in pure culture by using conventional cultivation approaches, indicating that the CORKs provide outstanding opportunities for retrieving microbes from this hard-to-access environment.

Porous oceanic crust may represent a significant microbial biosphere, which is often referred to as the "subseafloor ocean" (5, 12, 17, 30). Yet microbial life within oceanic crust remains largely unknown due to the difficulty of recovering intact, contamination-free oceanic crust. In hydrologically active regions, fluids emanating from crust have yielded important clues (6, 9, 23). Hydrothermal circulation of large volumes of seawater occurs in two different regions, seafloor spreading centers and ridge flanks (30). Based on evidence from various deep-sea hydrothermal systems located in the seafloor spreading centers, there is a growing consensus that the hydrothermal circulation of seawater and concomitant supply of inorganic energy and carbon sources might sustain a prosperous subseafloor microbial ecosystem (9, 19, 22, 41, 59). In seafloor spreading centers, hydrothermal alteration completely removes potential electron acceptors for microbial energy metabolism from intruded seawater and instead provides volatile energy and carbon sources (e.g., H_2 and CO_2). Therefore, thermophilic methanogens and thermophilic fermenters are likely to be the dominant organisms in habitats supported by altered hot fluids (9, 59).

In ridge flank crusts, the fluid-rock interaction and the supply of magmatic volatile species are less intense, and significant concentrations of electron acceptors, such as sulfate, have been found in circulating fluids (10, 66, 69). Similar fluids might exist even in crust in seafloor spreading centers where intruded seawater has not reached the high-temperature reaction zone. The flux of hydrothermal circulation through ridge flanks is increasingly recognized to be much greater than that in seafloor spreading centers (13, 39, 68). Therefore, hydrothermal circulation in warm ridge flank crusts may produce significant chemical fluxes to the overlying ocean (66, 68). Microbial effects on this potentially important geochemical process are poorly understood. The isolates obtained in this study are useful for evaluating the microbial roles in the warm ridge flank crust.

Microbial communities in ridge flank crusts may provide a key to a better understanding of the propagation of microorganisms inhabiting deep-sea hydrothermal vent environments in seafloor spreading centers. In vent fields located in seafloor spreading centers, there are cosmopolitan microbes, including members of the *Thermococcales* (hyperthermophilic fermenters), *Archaeoglobales* (mainly hyperthermophilic sulfate reducers), *Aquificales* (thermophilic hydrogen and/or sulfur oxidizers), *Methanococcales* (mesophilic to hyperthermophilic methanogens), and epsilon-*Proteobacteria* (mesophilic to thermophilic hydrogen and/or sulfur oxidizers) (40), suggesting that these microbial populations are less geographically isolated than macrofaunal communities living together (64). Especially, members of the *Thermococcales* have been found even in low-temperature diffusing fluids, suggesting that they are distributed globally in hot subseafloor environments (9, 19, 22). Although little is known about how these extremophiles could be distributed globally, hydrothermal circulation in ridge flanks is a potential stepping stone for the dispersal of microbial populations. The absence of these microorganisms, except for members of the *Methanococcales* and *Archaeoglobales*, was thus highly surprising. The most dominant members of the *Bacteria* and *Archaea* detected in the microbial community characterized in this study were *Ammonifex* group bacteria (potentially thermophilic sulfate reducers) and *Methanothermococcus* (thermophilic methanogens), respectively. Although more investigations of ridge flanks are necessary for further verification, recent studies, including this report, strongly suggest that oceanic crusts in hydrologically active ridge flanks might harbor subseafloor microbial communities that are distinct from the communities in pelagic sediments or crusts in seafloor spreading centers (6, 31).

The $\delta^{13}C_{VPDB}$ value of total organic carbon in the bulk sample was $-22.1‰$, which was ^{13}C depleted relative to both CO_2 dissolved in seawater (1‰) and CO_2 in typical vent fluids in natural deep-sea hydrothermal fields (-1 to $-10‰$) (51).

The energy sources available in hot ridge flank crustal fluids have been a subject of debate. Two different potential energy sources have been proposed: (i) organic matter released from sediments and (ii) H_2 produced by seawater-basalt reactions at high temperatures (6, 23). Considering that neither the isotopic fractionation nor the potential carbon fixation pathway of the most dominant phylotype, *Ammonifex* group bacteria, has been determined due to the resistance of the organisms to cultivation, the $\delta^{13}C_{VPDB}$ value should be interpreted with caution. However, the carbon isotopic fractionation to biomass measured for *A. degensii* grown chemolithoautotrophically was reported to be only 2.8‰ (21). In addition, the concentration of total CO_2 in fluids circulating through this ridge flank is very low (66, 69), suggesting that CO_2 in the effluent fluids might be unable to serve as the major carbon source for the microbial community characterized. Considering that the $\delta^{13}C_{VPDB}$ value of the total organic carbon in the sample was comparable to that in ocean sediments (−27 to ca. −20 ‰) (51), it is most likely that the major carbon source is photosynthesis-derived organic matter released from sediments. Even in a CO_2-rich hydrothermal field, it was recently demonstrated that photosynthesized organic matter released from deep sediments is the major carbon source of a bacterial mat (46).

It is well accepted that steel corrosion is stimulated by anaerobic microorganisms in the absence of oxygen (4), although the mechanism for this microbially influenced corrosion is still hotly debated. Molecular hydrogen, called cathodic hydrogen, which is formed on the steel surface by the dissociation of water, may serve as an energy source for hydrogen-oxidizing microorganisms, including sulfate reducers and methanogens (4). Especially sulfate reducers are notorious for stimulating steel corrosion by producing the corrosive agent hydrogen sulfide, either by utilizing cathodic hydrogen or organic matter as an energy source (4) or by directly utilizing electrons from steel (11). Although microbially influenced corrosion at high temperatures has been poorly investigated, the microbial community investigated in this study might be associated with the formation of black rust. Based on the results obtained in this study, we propose the following process for black rust formation and the concomitant succession of the microbial community: (i) colonization of microorganisms that are indigenous to ridge flank crust and are transported by effluent crustal fluids (precipitation of silicate from the hot fluids might help the microbial colonization [27]), (ii) activity of sulfate reducers, including *Ammonifex* group bacteria, is stimulated by sulfate supplied from bottom seawater, and (iii) increased production of hydrogen sulfide creates reducing microhabitats where methanogens can grow. This process might explain the absence of detectable methanogens in the effluent fluids (6, 23).

Much remains to be learned about biogeochemical processes within warm ridge flank oceanic crusts. The vertical distribution and horizontal distribution of microorganisms within ridge flank oceanic crusts have been poorly explored. The minimum energy, carbon, and nitrogen fluxes and the sources required to sustain microbial activities within the ridge flank crusts are of considerable interest but remain to be determined. Both further explorations of this substantial biosphere and further physiological characterizations of the retrieved microorganisms under in situ conditions are necessary.

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