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# Soil Microbial Community Structure across a Thermal Gradient following a Geothermal Heating Event

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**In this study microbial species diversity was assessed across a landscape in Yellowstone National Park, where an abrupt increase in soil temperature had occurred due to recent geothermal activity. Soil temperatures were measured, and samples were taken across a temperature gradient (35 to 65°C at a 15-cm depth) that spanned geothermally disturbed and unimpacted soils; thermally perturbed soils were visually apparent by the occurrence of dead or dying lodgepole pine trees. Changes in soil microbial diversity across the temperature gradient were qualitatively assessed based on 16S rRNA sequence variation as detected by denaturing gradient gel electrophoresis (DGGE) using both ribosomal DNA (rDNA) and rRNA as PCR templates and primers specific for the *Bacteria* or *Archaea* domain. The impact of the major heating disturbance was apparent in that DGGE profiles from heated soils appeared less complex than those from the unaffected soils. Phylogenetic analysis of a bacterial 16S rDNA PCR clone library from a recently heated soil showed that a majority of the clones belonged to the *Acidobacterium* (51%) and *Planctomyces* (18%) divisions. Agar plate counts of soil suspensions cultured on dilute yeast extract and R2A agar media incubated at 25 or 50°C revealed that thermophile populations were two to three orders of magnitude greater in the recently heated soil. A soil microcosm laboratory experiment simulated the geothermal heating event. As determined by both RNA- and DNA-based PCR coupled with DGGE, changes in community structure (marked change in the DGGE profile) of soils incubated at 50°C occurred within 1 week and appeared to stabilize after 3 weeks. The results of our molecular and culture data suggest that thermophiles or thermotolerant species are randomly distributed in this area within Yellowstone National Park and that localized thermal activity selects for them.**

Due to the inability to predict when major natural environmental changes may occur, microbial ecology studies typically examine climax communities that result from long periods of selection brought on by the last major shift in nutrient or physical conditions. Seasonal variation no doubt disturbs community equilibrium populations, but such changes are typically somewhat gradual and do not represent sudden and lethal events. Microbial ecologists seldom have the opportunity to study microbial community responses to dramatic, naturally occurring disturbances of any single key environmental factor. The present report describes studies that have taken advantage of a unique opportunity to examine a soil microbial community soon after a sudden temperature shift resulting from changes in the underlying geothermal activity.

Norris Geyser Basin is a highly active geothermal area located in the northwest section of Yellowstone National Park, Wyo. It comprises the southernmost part of a fault system that extends north to Mammoth Hot Springs and also lies at the edge of the most recent 0.6-Ma caldera of the Yellowstone plateau (6). Frequent changes in the landscape and thermal features in Norris Geyser Basin provide evidence of the active geothermal system that lies beneath. The research site in this study is located atop a glacially formed moraine known as "Ragged Hills." The expansion of the underlying, near-surface geothermal activity at this location was indicated by the sudden death of lodgepole pines (*Pinus contorta*) in a well-defined area. This observation was a visual cue to recent increases in soil temperature; disease or insect damage to trees is typically manifested by gradual (weeks-to-months) change in tree phenotype. Measurements at the site confirmed that the soil temperatures in the area delineated by the dead trees were significantly elevated relative to those of immediately adjacent soils underlying green, live trees. This event provided a natural system in which to study the effects of a prominent environmental variable, temperature, on microbial diversity in situ.

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## MATERIALS AND METHODS

**Field site and soil analysis.** The elevation of Ragged Hills is roughly 100 m above the geyser basin floor. The lodgepole pine trees at this site (live or dead) were of uniform age, dating from the regrowth that followed the large summer fire of 1988 in Yellowstone National Park, which decimated the stand of trees on this hill and much of the surrounding region. Tree stand death was first observed during the third week of July 1999. Elevated soil temperatures were then verified. Detailed soil temperature measurements were taken on 18 October 1999 at a depth of 15 cm by using a hand-held thermometer. Based on these measurements, a sampling transect was established across an area that included heat-impacted and unaffected soil.

Chemical analysis of soil samples taken from the sites used for molecular analysis was carried out to examine other major selection factors that could potentially influence the microbial community across the landscape. Total organic carbon and pH were measured at each site. Also, primary dissolved inorganic constituents of a saturated paste extract from each transect location were determined using either inductively coupled plasma emission spectrophotometry or ion chromatography. All analytical methods have been described previously (23, 28).

**Plate count analysis of soil populations.** Soil samples were collected to estimate the number of viable thermophiles and mesophiles present at specific

locations in the transect. For sites B and F, 5 g of soil was suspended in 45 ml of sterile phosphate-buffered saline (137 mM NaCl, 2.5 mM $KH_2PO_4$ 6.9 mM $K_2HPO_4$ [pH 7.3]) and shaken for 1 h. Coarse soil particles were allowed to settle for 5 min, the soil suspensions were serially diluted (10-fold steps) into phosphate-buffered saline, and 100-μl aliquots of each dilution were plated in triplicate onto R2A (Difco) (D. J. Reasoner and E. E. Geldreich, Abstr. 79th Annu. Meet. Am. Soc. Microbiol. 1979, abstr. N7, 1979) and 0.1% yeast extract agar media. Colonies were counted after incubation for 1 week at either 25 or 50°C.

**Nucleic acid extraction.** Soil samples for molecular analysis were also taken at each established 3-m interval site within the sampling grid. Samples were collected aseptically to a depth of 15 cm by using autoclaved polyvinyl chloride (PVC) coring devices. The coring devices were simple 2.54-cm-diameter PVC pipes that had been cut at an angle on one end to provide a sharpened end to aid in soil penetration. The soil in the Ragged Hills area was gravelly and porous to the 15-cm depth at which samples were collected. Individual soil samples were homogenized and stored at −80°C. DNA and RNA were extracted by the method of Purdy et al. (25) with some modifications. Briefly, 0.5-g soil samples were measured into 2-ml screw cap tubes containing 0.5 g of zirconium beads (Biospec Products Inc., Bartlesville, Okla.), 0.7 ml of 120 mM sodium phosphate (pH 7.7) plus 1% (wt/vol) acid-washed polyvinylpolypyrrolidone, 0.5 ml of Tris-equilibrated phenol (pH 8.0), and 50 μl of 20% (wt/vol) sodium dodecyl sulfate. A Bio 101 Fast Prep bead beater was used to lyse the samples at 6.5 m/s for 45 s. The samples were then centrifuged at 13,000 × *g* for 15 min. Supernatants from two duplicate samples were pooled and loaded in two 0.7-ml aliquots onto hydroxyapatite (HTP) spin columns, which were made by packing 1-ml plastic syringes with 0.7 ml of resin. The loaded spin columns were centrifuged in a swing-bucket rotor at 100 × *g* and 25°C for 4 min. After centrifugation, the columns were washed three times with 0.5 ml of 120 mM $Na_2HPO_4$ (pH 7.0). RNA was eluted from each column using three 0.7-ml washes with 140 mM $K_2HPO_4$ (pH 7.2) and collected as fractions in sterile 1.5-ml tubes. The first fraction, which contained approximately 90% of the RNA, was precipitated overnight at −20°C with the addition of 0.5 volume of 7.5 M LiCl–50 mM EDTA. After RNA elution, DNA was eluted from the columns with 0.4 ml of 300 mM $K_2HPO_4$ (pH 7.2) and precipitated overnight at −20°C with 2.5 volumes of ethanol and 1/10 volume of 3 M ammonium acetate. RNA and DNA pellets were suspended in 50 μl of nuclease-free water and desalted using Micro-Spin G-25 columns (Supelco, Bellefonte, Pa.). The samples were then precipitated overnight at 4°C with 1/2 volume of polyethylene glycol 8000, 1/10 volume of 5 M NaCl, and 1 μl of 20-mg/ml glycogen. The pellets were suspended in 100 mM Tris–10 mM EDTA (TE buffer) and stored at −70°C (RNA) or −20°C (DNA). The yield and quality of nucleic acid preparations were checked by comparison with standards on a 1% agarose gel.

**Nucleic acid amplification.** PCR amplification of domain *Bacteria* 16S rRNA genes from community DNA for denaturing gradient gel electrophoresis (DGGE) analysis was done using the *Bacteria*-specific forward primer 1070F (10) and the universal reverse primer 1392R containing a GC-clamp (2). Each reaction mixture contained 2.5 mM $MgCl_2$, 0.2 mM each of the four deoxynucleoside triphosphates (dNTPs), 0.5 μM (each) primer, approximately 10 ng of template DNA, 0.5 mg of bovine serum albumin per ml, 2.5 U of *Taq* polymerase per ml, and 1× buffer (Promega, Madison, Wis.) in a total volume of 50 μl. The PCR amplification cycle was as follows: 2 min at 95°C, then 24 cycles of 45 s of denaturation at 95°C, 45 s of annealing at 43°C, and 45 s of extension at 72°C, followed by a final extension of 7 min at 72°C. Reaction mixes and the thermocycler program for the PCR amplification of archaeal 16S rRNA sequences were the same as for the bacterial reactions, except that the domain *Archaea*-specific forward primer A931F (2) was used with an annealing temperature of 56°C and 26 cycles of amplification.

Reverse transcriptase PCR (RT-PCR) amplification of RNA templates was accomplished in a two-part reaction. Reverse transcription was carried out for 60 min at 37°C in a 20-μl reaction mix containing 0.5 mM (each) dNTP, 1 μM (each) primer 1070F and 1392R, and 1 μl of Sensiscript RT (S-RT) (Qiagen, Valencia, Calif.). RNA was treated with RQ1 DNase prior to RT-PCR. The absence of DNA contamination was verified by assembling the same reactions without S-RT. PCR amplifications were then done using 5 μl of the reverse transcription reaction product as template and the same reaction conditions given above. PCR (or RT-PCR) was conducted in a Perkin-Elmer model 9700 thermocycler, and products were quantified on a 1% agarose gel by comparison to mass ladder standards (GibcoBRL, Grand Island, N.Y). Approximately 120 ng of PCR product per lane was loaded onto the DGGE gels. Initial extraction and amplification experiments were done in triplicate to rule out variability in the procedure.

**DGGE analysis.** DGGE was performed using a DCode system (Bio-Rad, Hercules, Calif.) as described by Muyzer et al. (17). An 8% polyacrylamide gel with a linear denaturant concentration from 40 to 70% (where 100% denaturant contains 7 M urea and 40% [vol/vol] formamide) was used to separate the PCR products obtained as outlined above. The gels were electrophoresed for 17 h at 60°C and a constant 60 V, stained for 30 min with SYBR Green I (Molecular Probes, Eugene Org.), illuminated on a transilluminator, and photographed using Polaroid 57 film.

**Clone library construction.** To obtain more descriptive information about the soil microbial community, a *Bacteria* clone library was constructed for sequence and phylogenetic analysis. Near-full-length rDNA clones were amplified using total DNA extracted from site B, along with *Bacteria*-specific primers 8F and the universal primer 1492R (2). PCR reagents were used at the same concentrations as in the bacterial PCR amplifications described above for DGGE analysis. The amplification program consisted of 2 min at 95°C, then 30 cycles of 1 min of denaturation at 95°C, 1 min of annealing at 45°C, and 1 min of extension at 72°C, followed by a final extension of 7 min at 72°C. For amplification of DNA from culture isolates, the same reaction and amplification conditions were used except that instead of using 2 μl of template DNA, individual colonies were stabbed with a sterile toothpick and rinsed into a prepared reaction mix just prior to PCR amplification. Also, a 10-min cell lysis and denaturation step at 95°C was added at the start of the amplification program. PCR products were purified from a low-melting-point agarose gel with Amicon microcon filtration devices (Millipore, Bedford, Mass.) and then cloned in pCR2.1-TOPO and transformed into *Escherichia coli* TOP10 as described by the manufacturer (Invitrogen). A total of 192 clones were screened with the enzymes *Rsa*I and *Hae*III to generate restriction fragment length polymorphism (RFLP) groups, representatives of which were sequenced for phylogenetic analysis.

**Sequencing and phylogenetic analysis.** Sequencing was accomplished using the ABI Prism BigDye Terminator cycle-sequencing reaction kit and an ABI 310 DNA sequencer (Perkin-Elmer, Norwalk, Conn.). Primers used for sequencing were targeted to vector plasmid sequences flanking the multiple-cloning site, M13F and M13R, and also to regions within the cloned fragments. The internal primers included 1070F (10), 338F, 338R, 522F, 522R, and 785F (2, 13). A single sequencing reaction was performed for clones representing each of the 39 RFLP-designated groups to determine if the RFLP groupings represented different operational taxonomic units (OTUs). A BLAST search of the GenBank database was conducted to identify the nearest relatives of these 39 partially sequenced clones (*E. coli* positions 340 to 700). Of this group, 10 were selected for near full-length sequencing. Additionally, all of the pure culture isolates (a total of five) from cultivation experiments incubated at 50°C were chosen for 16S rDNA sequencing. Sequences were screened for chimeras using the CHECK_CHIMERA program of the Ribosome Database Project and by manual alignments of secondary structure. As a final check for chimeras, each sequence was split into 5′ and 3′ fragments, which were analyzed separately by BLAST searching of GenBank. Sequences for which either the 5′ or 3′ fragment had significantly different closest relatives were considered probable chimeras and were removed from the data set. Phylogenetic analysis was done using the ARB software package (http://www.mikro.biologie.tu-muenchen.de/pub/ARB/). Phylogenetic trees of the manually aligned sequences were constructed using parsimony/maximum-likelihood, DNA ML (Fast DnaML), and neighbor-joining treeing programs with *Aquifex pyrophilus* and *Thermotoga maritima* used as the outgroups.

**Soil microcosm experiment.** Soil was collected in a sterile container from the vicinity of site F (unperturbed by the heating event) in October 2000, taking soil to the same depth (15 cm) as for the original soil transect samples. The soil temperature at that time was 24°C at 15 cm deep. The soil was transported back to the laboratory and aseptically homogenized, and then 30 g of soil was added to each of 12 sterile 50-ml polypropylene tubes (Falcon tubes). Four replicate tubes were incubated in water baths at temperatures of 25 or 50°C. Tube caps were unscrewed slightly to maintain an aerobic headspace without compromising sterility. The tubes were weighed daily, and sterile water was added as necessary to maintain a constant soil moisture content. At weekly intervals, 3 g of soil in 0.25-g aliquots was removed from each tube and immediately frozen at −80°C for subsequent analysis.

A faster, alternative method for nucleic acid purification was used with these samples. Both RNA and DNA were purified from soil samples by a method adapted from that of Schmidt-Goff and Federspiel (26). To each 0.25-g sample, 250 μl of water was added and samples were flash frozen. Then 0.5 g of zirconium beads, 33.3 μl of 20% sodium dodecyl sulfate, 167 μl of 3% diatomaceous earth (Sigma, St. Louis, Mo.), and 583 μl of Tris-buffered phenol were added to the still frozen soil slurry samples. Samples were quick thawed by placing the tube in warm water, and then each sample was immediately shaken for 160 s on a bead mill and centrifuged for 15 min at 4°C and 13,000 × *g*. The aqueous layer was transferred to a fresh tube, and nucleic acids were precipitated at −20°C with 3

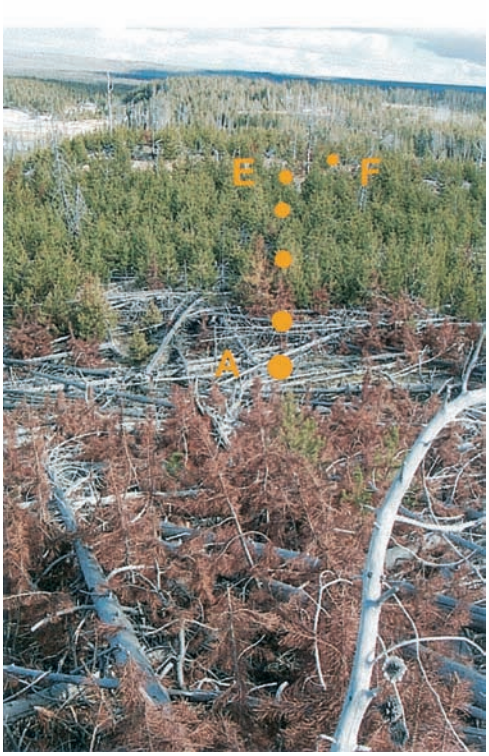


FIG. 1. View of the Ragged Hills research site in Norris Geyser Basin, Yellowstone National Park, Wyo. Red needles are seen on lodgepole pines in the foreground, where recent geothermal activity caused heat killing. At the time of the photograph was taken, a few dead or dying trees retained a few green branches even though the majority of the tree was brown. A sampling transect was established across this site, from foreground to background in the photo.

M sodium acetate (pH 5.2) and 95% ethanol. After centrifugation (13,000 × *g*), the nucleic acid pellet was washed with 70% ethanol and suspended in 25 μl of nuclease-free water, and four individual samples were combined. The RNA was further purified and DNase treated using the SV total RNA isolation system (Promega) as described by the manufacturer. DNA was also purified using the SV total RNA isolation system by substituting an RNase step for the DNase treatment. Purified DNA or RNA was eluted from the SV column with 100 μl of nuclease-free water, and DNA (or RNA) integrity was verified in agarose gels. PCR or RT-PCR and DGGE analysis were performed as described above. DGGE bands were purified and sequenced as described elsewhere (21).

**Nucleotide sequence accession numbers.** The 16S ribosomal DNA (rDNA) sequences from the clone library were submitted to GenBank and can be found as accession numbers AF465644 to AF465659. DGGE band sequences were given GenBank accession numbers AF465660 to AF465678.

## RESULTS

**Soil temperature and chemistry.** Trees with red needles were either dead or dying as a result of elevated soil temperatures in the rooting zone of these trees and clearly marked the point of the temperature transition (Fig. 1). Detailed soil temperature measurements taken on 18 October 1999 allowed the establishment of a transect wherein soil temperatures at a depth of 15 cm ranged from 65°C on one end of the transect (site A) to 35°C in the unaffected area of green trees (sites D, E, and F). Between these two extremes was a nearly linear temperature gradient (Fig. 2). Soil chemical constituents were analyzed in soils from the sites where samples were taken for

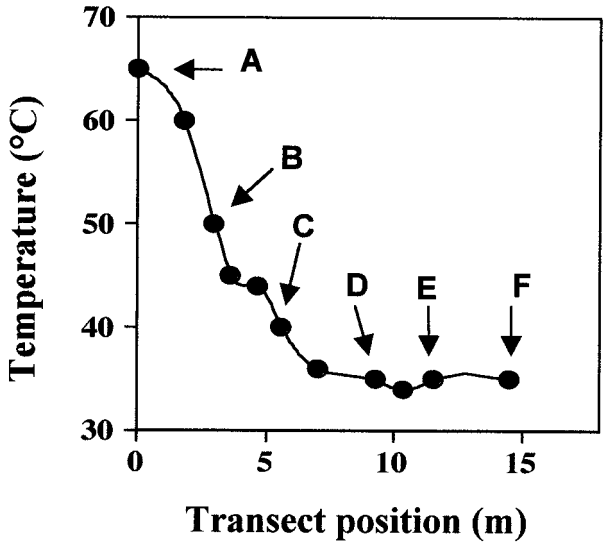


FIG. 2. Soil temperatures measured across the field site transect. Temperatures are shown for the 15-cm depth. Samples were collected for molecular analysis at sites corresponding to the letters in Fig. 1.

molecular analysis to assess whether there were other potential strong selection pressures that could influence microbial diversity and community structure at these sites (Table 1). Both ammonia and nitrate concentrations were significantly higher in the hottest soil site, A, than in the other sites. The electrical conductivity (EC) of the site A soil was also higher, and its pH differed significantly from the others. Other factors important for microbial growth, including phosphorus, organic matter content, and soil moisture content, were not significantly different between sites. Analysis for potassium and trace metals also showed no patterns corresponding to the observed temperature gradient. Values ranged as follows (in milligrams per kilogram of soil): K, 28 to 76; Cu, 0.18 to 0.38; Fe, 87 to 132; Cd, 0.02 to 0.06; Cr, <0.01 to 0.02; Ni, 0.04 to 0.12; Pb, 0.32 to 0.75; and Zn, 0.44 to 1.48.

**Community analysis using DGGE.** DNA and RNA were extracted from soil samples taken at sites designated in Fig. 2. PCR with the *Bacteria*-specific primer and a universal primer containing a GC-clamp and DNA purified from each site resulted in a single 350-bp fragment (the expected size). These PCR products were separated using DGGE to qualitatively

TABLE 1. Chemical analysis of soils sampled along the temperature gradient in the field transect established at Ragged Hills

| Site[a] | $NH_4^+$ concn (mg/kg) | $NO_3^-$ concn (mg/kg) | Phosphorus concn (mg/kg)[b] | EC (μS/cm) | % Organic matter | pH |
|---|---|---|---|---|---|---|
| A | 44.6 | 12.2 | 24.9 | 780 | 1.43 | 3.7 |
| B | 5.0 | 5.7 | 42.9 | 70 | 1.79 | 5.1 |
| C | 6.1 | 1.4 | 44.6 | 50 | 4.20 | 4.8 |
| D | 3.0 | 0.4 | 14.1 | 30 | 3.22 | 4.8 |
| E | 4.5 | 0.3 | 12.3 | 40 | 2.63 | 4.8 |
| F | 3.3 | <0.1 | 12.6 | 40 | 2.75 | 4.7 |

[a] Site designations correspond to positions depicted in Fig. 1.
[b] $NaHCO_3$-extractable phosphorus by the Olsen method.

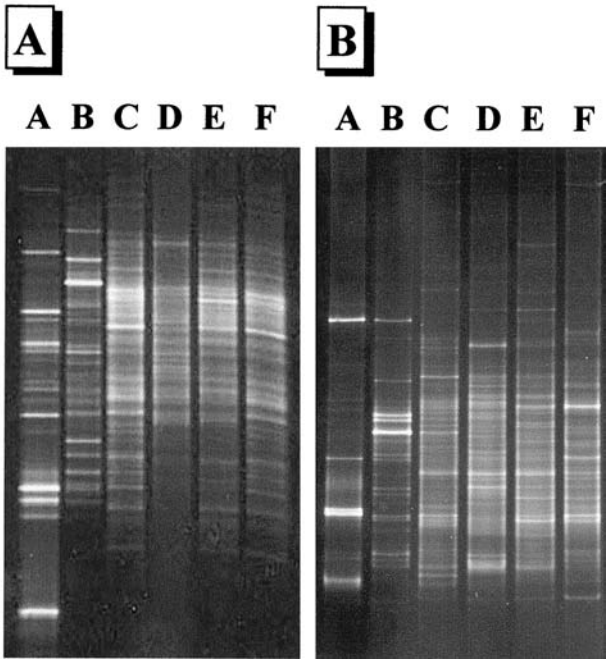


FIG. 3. DGGE analysis of microbial diversity across the field sampling temperature transect. (A) DGGE profiles based on 16S rDNA of bacterial communities at different sampling sites. Letters correspond to transect sites depicted in Fig. 1. Sites A and B are significantly heat-impacted sites, and C through F are not. (B) DGGE profiles of the archaeal communities in the same samples.

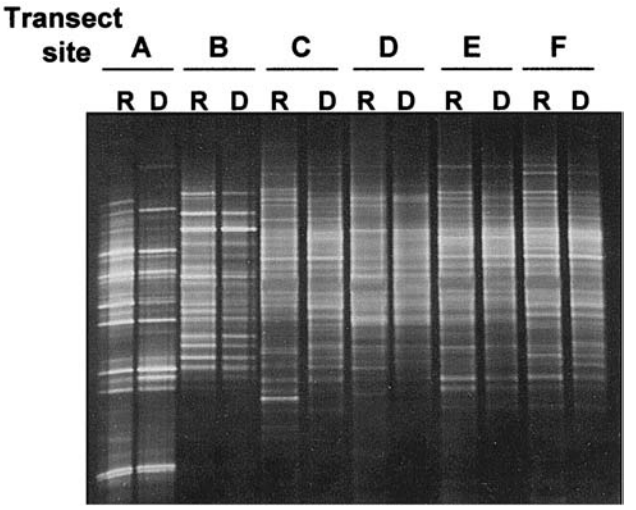


FIG. 4. Comparison of RNA and DNA as templates for DGGE analysis. RNA or DNA extracted from the same sample was used as the template for PCR to generate bacterial DGGE profiles. The profiles for each site are presented in pairs, with the RNA (R)-derived profile on the left and the DNA (D)-derived profile on the right. The transect sampling sites (A to F) are the same as those described for Fig. 2 and 3.

assess bacterial diversity at each soil site (Fig. 3A). The bacterial communities from each of these sites appeared potentially complex, but there were obvious differences between the heat-impacted (sites A and B) and unimpacted (C to F) sites. At the hottest location (site A, 65°C), there were roughly 10 prominent bands and about 10 minor bands. Site B, located on the very edge of the heat-impacted area, had a few additional bands. The DGGE profiles of both sites A and B were substantially simpler than those of the sites unaffected by the heating event, where the bands were too numerous to count and in some cases were merging. The archaeal members of the communities were examined in the same manner (Fig. 3B). The archaeal community profiles were analogous to those of the bacterial community profiles in that the heat-affected sites A and B had very few bands compared to the cooler sites. As with the bacterial DGGE profiles, the differences between sites B and C were from communities separated by only 3 m. RNA was also compared as an alternative PCR template for DGGE analysis. The RNA- and DNA-based bacterial DGGE profiles for each site are presented in adjacent lanes in Fig. 4. With a few minor exceptions, the RNA- and DNA-derived DGGE profiles were identical.

**Enumeration of soil bacteria.** An agar plate cultivation experiment was used to evaluate the relative abundance of thermophiles or thermotolerant species. Sites B and F were chosen to represent heat-impacted and nonimpacted soil, respectively, and were deemed more appropriate for examining thermophile distribution in this general area (e.g., as opposed to comparing sites B and C) because of concerns that spore transport from one location to another could confound interpretations. Duplicate sets of agar plates were incubated at either 25 or 50°C, with the incubation temperature representing an average soil temperature measured during the entire course of the study for sites F and B, respectively. When incubated at 25°C, soil dilutions from site B yielded viable-count estimates (all shown as $10^5$/cells per gram of soil) of 5.2 ± 0.4 on R2A agar and 8.7 ± 0.7 on 0.1% yeast extract, whereas site F colony counts were 60 ± 10 on R2A agar and 77 ± 4 on 0.1% yeast extract. At 50°C, colony counts for site B were 4.5 ± 0.1 on R2A agar and 9.1 ± 0.2 on 0.1% yeast extract, while site F colony counts were 0.011 ± 0.002 on R2A agar and 0.003 ± 0.0003 on 0.1% yeast extract. Thus, site F, which was not heat impacted, yielded roughly fivefold-higher colony counts than did site B, the heat-impacted site, on both types of agar media incubated at 25°C. At 50°C, however, site B had colony counts that were two orders of magnitude greater than those at site F, suggesting that populations of thermophilic species were present in substantially larger numbers in the heat-impacted soil. Different colony types (based on visual selection) from incubations at 50°C were subcultured to purity for 16S analysis. From site B, the majority of colonies were of a single type, brown with an actinomycete colony appearance and composed of cells exhibiting actinomycete morphology. Microscopic inspection of the other isolates demonstrated gram-positive, spore-forming cells, essentially defining organisms belonging to *Bacillus* or recently described branch genera (19).

**Clone library construction and phylogenetic distribution of clones.** A 16S rDNA analysis was conducted to identify the bacterial species that were apparently selected for by geothermal soil heating. Site B was chosen for the analysis because it was on the very edge of what appeared to be a thermal spreading zone (immediately under a heat-killed tree) and was the first site along the temperature gradient in which the apparent

diversity was notably reduced in the DGGE analysis. Thus, the populations present at this site at the time of sampling probably represented a transitional community that would include thermophile populations that are thriving subsequent to the heating event and perhaps remnants of some mesophile populations that were on the decline. As a first approximation for phylogenetic grouping and to help direct sequencing efforts, 196 near-full-length clones were subjected to RFLP analysis. Digests with *Rsa*I and *Hae*III generated 5 OTUs to which four or more clones were assigned and accounting for a total of 80 clones, and ten additional OTUs containing 2 or 3 clones each, comprising an additional 21 clones. The remaining clones generated unique RFLP patterns.

Partial sequencing of approximately 350 bp, which included the V6 variable region (based on the *E. coli* sequence), was done on 39 clones representing all of the major OTUs plus additional unique OTUs. The largest group, composed of 20 clones (or 51% of the total), most closely matched the *Acidobacterium* group (89 to 98% similarity). The next largest OTU was best represented by the *Planctomyces* group (91 to 95% similarity), which included seven clones (18% of the total). The α *Proteobacteria* group constituted another 13% (five clones), and the remaining clones were members of the low-G+C gram-positive bacteria (4 clones), γ *Proteobacteria* (one clone), and *Prosthecobacter* (one clone) and *Actinomycete* (one clone) groups. At least one representative clone from each of these OTUs was chosen for near-full-length sequencing to accommodate a more extensive phylogentic analysis and comparison with bacteria described for other soils. For the larger groups, multiple sequences were chosen so that the phylogenetic affiliation of the sequenced clones was reflective of the distribution in the initial RFLP/sequence analysis. The five colony types obtained in the cultivation experiments were also chosen for full-length sequence analysis to compare results from the culture-based and molecular methods.

**Phylogenetic analysis.** Taxonomic assignment of the clones did not change as a result of full-length analysis. Three methods of tree construction were used in this study: maximum-likelihood, maximum-parsimony, and neighbor-joining. For simplicity, only the neighbor-joining tree is presented in Fig. 5. The sequences were distributed into seven different groups with strong bootstrap support. Within each of these groups, there was very high bootstrap support to nearest relatives as well. The topology of the alternative trees differed somewhat in the branching of the major groups. An example is the placement of the *Planctomyces* group. In the neighbor-joining tree, the *Prosthecobacter* and *Planctomyces* groups had a common node that diverged from the *Acidobacterium* group, while in the maximum-likelihood and maximum-parsimony trees, the *Prosthecobacter* and *Acidobacterium* groups had a common node, which diverged greatly from the *Planctomyces* group. More importantly, however, the topologies within the major groups were identical for each tree.

For most of the clone library sequences, the nearest relatives were uncultivated bacteria from other soil studies, some of which are presented in the tree. The majority of the uncultivated clones in this study belonged to the acidobacteria, with clones 46A and 72A being most closely related to *Acidobacterium capsulatum*, one of only three cultivated members of this group. The other two acidobacterial clones, 2B and 5A, were each more closely related to soil clones from The Netherlands or Australia than to each other, with 100% bootstrap support.

Four of the five cultivated bacteria clustered in the *Bacillus* group of the low-G+C gram-positive bacteria. The branches within this group are highly supported, and in general the sequence similarity of the cultivated clones to known sequences was much higher than that of the uncultivated clones. Three of these sequences have 99% identity to previously cultivated bacteria. One of the uncultured clone sequences, clone 70A, also falls into the *Bacillus* cluster. However, clone 70A is divergent from the cultured *Bacillus* clones obtained in this study with a branch point having 93% bootstrap support. As might be expected, several of the close relatives of the clones in this group are known thermophilic species, such as *Bacillus stearothermophilus* and *Bacillus thermodenitrificans.* The remaining cultivated clone 1P-1 is a member of the high-G+C gram-positive bacteria and is the only clone in this study belonging to this group.

**Soil microcosm experiment.** The combined studies of field samples suggested that thermophiles were present at low levels in nonthermal soils of the Ragged Hills area and were selected for as a result of the geothermal heating event. To assess the sensitivity of the molecular methods of this study for detecting changes in the microbial community in response to heating and to examine the temporal component of the change, a laboratory experiment was designed to simulate the conditions of such a geothermal heating event (see Materials and Methods). DGGE analysis was based on the RNA template (Fig. 6). For samples incubated at 25°C (Fig. 6, compare lane 1 to lanes 2 to 5), there was little or no apparent change in community structure over the course of the 4-week experiment. In contrast to the 25°C samples, there were significant changes in the soils incubated at 50°C (Fig. 6, lanes 6 to 9). After just 1 week, additional bands (lane 6) marked the profile, an indication that heating of the soil was a selective force influencing the community structure. This profile may represent transitional populations, however, because by the second week (lane 7), the prominent bands were observed to occur further down in the denaturing gradient and the profile appeared stabilized with only a few additional changes in weeks 3 and 4 (lanes 8 and 9).

The DGGE profiles of the 50°C samples were also compared to that of site B from the soil transect, which most closely corresponded in temperature (Fig. 6, compare lanes 9 and 10). The profiles obtained were clearly different, but they did share a few bands with identical migration distance, albeit different intensity. Overall, though, the similarity lies in the fact that both have fewer total bands than do profiles from soils that were not heated.

Prominent bands in the 50°C soil microcosm profile and similarly migrating (primarily) bands in the site B DGGE profile (all indicated by dots in Fig. 6) were purified and sequenced to identify potential thermophiles and to determine if the profiles had common sequences (Table 2). Of those successfully purified, the band sequences from site B were similar to the clone library sequences in their phylogenetic distribution within various divisions of the *Bacteria* domain. Three of the eight bands had nearest relatives from the Ragged Hills clone library sequences, with two of these being identical matches (bands 13 and 16). Two other bands, bands 14 and 20, also were close relatives of clone library sequences (97% identical)

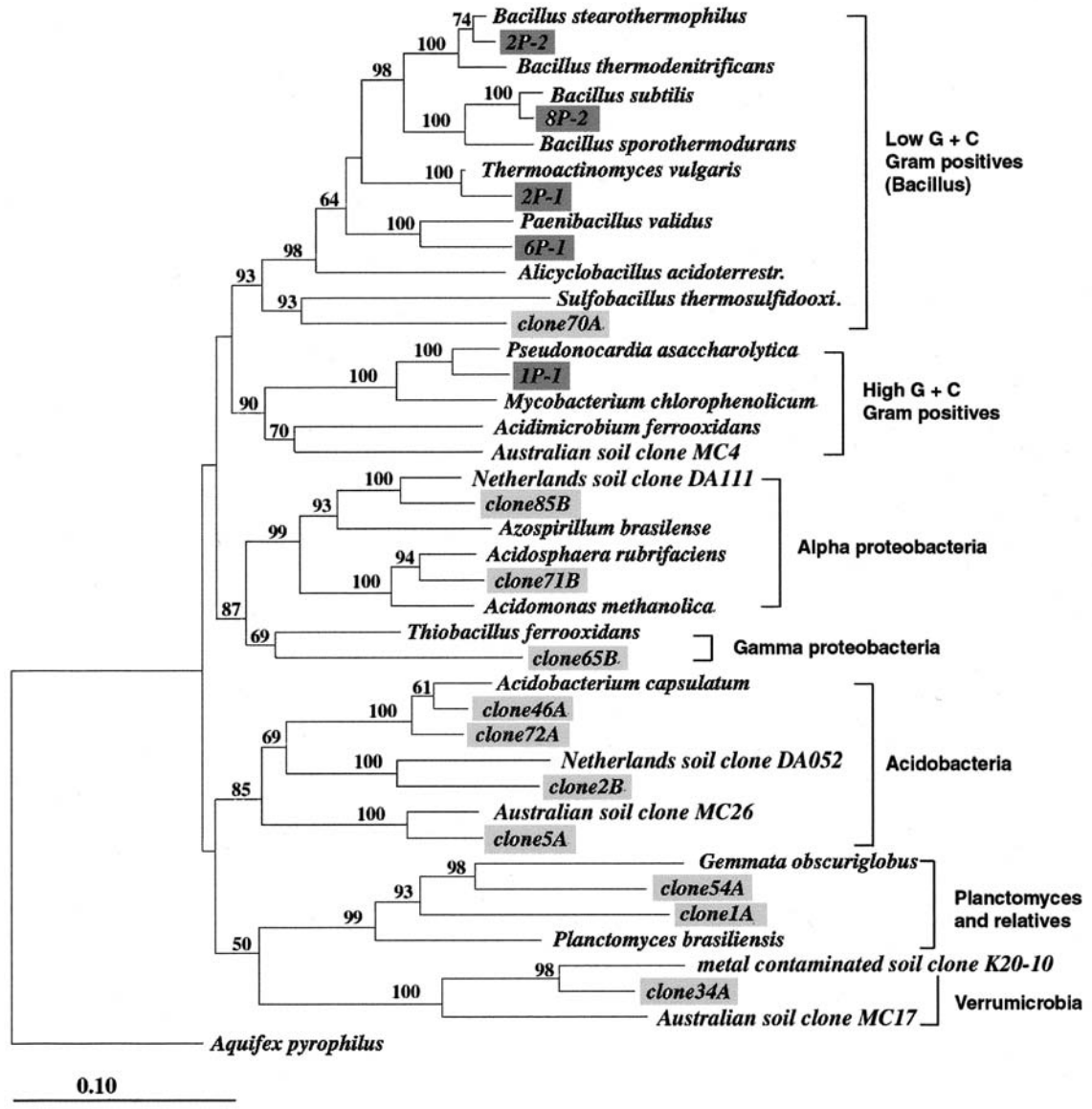


FIG. 5. Phylogenetic dendrogram of 16S rDNA bacterial sequences PCR amplified from nucleic acids extracted from Ragged Hills soil. Dark grey boxes indicate bacterial isolates, and light grey boxes indicate sequences of PCR-amplified community clones. The neighbor-joining tree was constructed with the ARB software package by using the Jukes and Cantor algorithm. Numbers at branch points represent confidence levels of 5,000 bootstrap pseudoreplicates. Only bootstrap values above 50% are shown. The scale bar represents 0.1 change/site.

but are not listed as such in Table 2, where only the nearest relative is given. In contrast, band sequences from the 50°C soil from the microcosm experiment had a rather different distribution. Roughly half of these sequences (11 altogether) belonged to the *Alicyclobacillus* genus. However, nearest relatives from the 50°C soil microcosm also included one of the Ragged Hills soil isolates from this study, a sequence from the site B clone library, and a sequence obtained from another thermal soil in Yellowstone National Park. Interestingly, the nearest relative of one band from the 50°C soil microcosm was one of the DGGE bands from Ragged Hills site B, although with only 91% identity. Although the phylogenetic distribution of sequences that were selected for in the 50°C soil microcosm samples was different from those obtained directly from field samples, clearly there were also some sequences with very close identity to those retrieved from the heat-impacted site.

Also of interest in this experiment, both DNA and RNA were purified from the microcosm soil samples by using a different, more rapid method from that used with the initial field samples, so it was necessary to compare the results obtained with each method to verify that the differences between field sample profiles and those obtained from the microcosm work were not due to the method. To demonstrate that the two methods provided equal lysis efficiency, DGGE profiles of RT-PCR products derived from RNA extracted by the two methods were compared (Fig. 6, lanes 10 and 11). Profiles in each lane were identical, indicating that the two methods were equivalent.

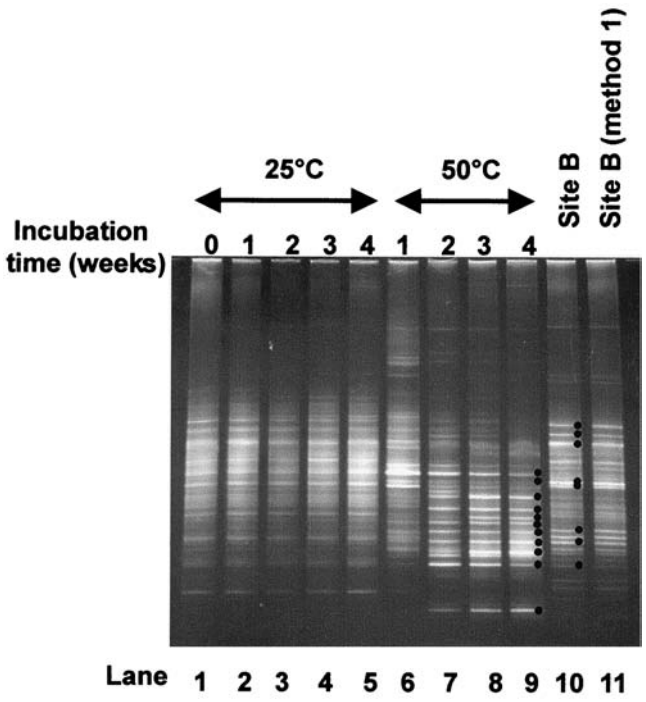


FIG. 6. RT-PCR DGGE analysis of soil microcosm experiment. DGGE profiles for soil starting material (lane 1) (for both temperature treatments) and samples incubated at 25°C (lanes 2 to 5) or 50°C (lanes 6 to 9) are shown. For site B, DGGE profiles utilized RNA purified by the hydroxyapatite method (lane 10) or the Promega SV total RNA kit (method 1 in lane 11). Dots indicate bands that were reamplified, purified, and sequenced.

It was of interest to determine if in response to a major perturbation, apparent differences in DNA and RNA synthesis and content could be detected using (RT-)PCR and DGGE. Community DGGE profiles derived from the starting material with the two different nucleic acid templates were found to be very similar in terms of band number and position, although differences in band intensity were noted (Fig. 7). After 1 week of heat treatment, however, both DNA- and RNA-based profiles were very different from those of the starting material but also differed from each other. By week 2, the DGGE profiles derived from both RNA and DNA were quite similar, and by week 3, they were almost identical in number and position of bands, although the band intensity did vary somewhat for a few bands. After an additional week of incubation, a few additional changes occurred in both RNA- and DNA-based DGGE profiles that somewhat reduced the overall apparent similarity of these community diversity estimates.

## DISCUSSION

The geothermal event at Ragged Hills offered a rare opportunity to examine the impact of a major environmental perturbation on a naturally occurring soil microbial community. Being able to study community responses in situ, soon after onset and without manipulation of the environmental conditions, allowed us to examine survivors and/or early colonists in a natural setting. Evidence that the changes observed in the microbial community were due to temperature effects came from visual inspection of the lodgepole pines and correlation

TABLE 2. Nearest GenBank relatives of organisms represented by 16S rRNA DGGE band sequences

| Soil and band | Sequence length (bp) | GenBank accession no. | Nearest GenBank relative | Accession no. of relative | % Identity |
|---|---|---|---|---|---|
| 50°C soil microcosm | | | | | |
| 1 | 323 | AF465660 | *Alicyclobacillus cycloheptanicus* | AB042059 | 100.0 |
| 2 | 320 | AF465661 | *Alicyclobacillus cycloheptanicus* | AB042059 | 99.1 |
| 3 | 320 | AF465662 | *Alicyclobacillus hesperidum* | AJ133633 | 99.7 |
| 4 | 333 | AF465663 | *Actinobacterium* (isolate from Ragged Hills [this study]) | AF465644 | 99.4 |
| 5 | 310 | AF465664 | Uncultured α-proteobacterium (Ragged Hills clone library [this study]) | AF465654 | 99.7 |
| 6 | 336 | AF465665 | Uncultivated bacterium (uranium mining waste pile) | AJ295647 | 96.4 |
| 7 | 278 | AF465666 | *Alicyclobacillus cycloheptanicus* | AB042059 | 98.5 |
| 8 | 312 | AF465667 | *Alicyclobacillus cycloheptanicus* | AB042059 | 98.1 |
| 9 | 316 | AF465668 | Uncultured bacterium DGGE band (this study) | AF465676 | 91.2 |
| 10 | 331 | AF465669 | Uncultured thermal soil bacterium clone (Yellowstone National Park) | AF391980 | 96.7 |
| 11 | 341 | AF465670 | *Sulfobacillus yellowstonensis* | AY007665 | 95.0 |
| Ragged Hills site B | | | | | |
| 12 | 317 | AF465671 | Uncultured γ-proteobacterium (Ragged Hills clone library [this study]) | AF465652 | 100.00 |
| 13 | 340 | AF465672 | Uncultivated eubacterium (polychlorinated biphenyl-polluted soil) | AJ292586 | 98.2 |
| 14 | 315 | AF465673 | Uncultured *Acidobacterium* group bacterium (Ragged Hills clone library [this study]) | AF465656 | 99.7 |
| 15 | 325 | AF465674 | Uncultured planctomycete (Yellowstone National Park thermal soil) | AF465657 | 100.0 |
| 16 | 210 | AF465675 | *Cellulomonas hominis* | X82598 | 95.7 |
| 17 | 317 | AF465676 | Uncultured bacterium DGGE band (Yellowstone National Park thermal soil) | AF465668 | 91.2 |
| 18 | 329 | AF465677 | Uncultivated bacterium clone (Yellowstone National Park thermal soil) | AF391988 | 97.3 |
| 19 | 322 | AF465678 | *Isosphaera* sp. | X81958 | 96.6 |

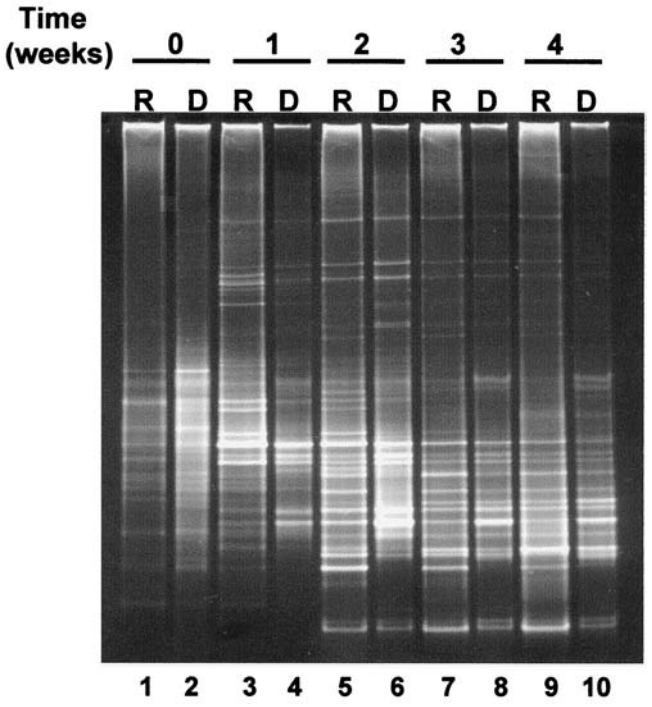


FIG. 7. Comparison of the RNA and DNA templates for DGGE analysis in the soil microcosm experiment. RNA and DNA were extracted from samples at the time points shown and used as templates for *Bacteria*-specific RT-PCR or PCR, respectively, the products of which were separated by DGGE. The DGGE profiles for each time point of the 50°C microcosm are presented in pairs, with RNA-derived profiles (R) on the left and DNA-derived profiles (D) on the right. Lane numbers are shown at the bottom.

with measured soil temperature (Fig. 1 and 2). Evidence of tree disease, insect damage, or severe nutrient or drought limitation was completely lacking. Rather, tree death occurred along well-defined lines and correlated perfectly with elevated soil temperatures measured in the area. Chemical analysis of the soils studied (Table 1) provided no significant evidence that other environmental factors imposed patterned selection effects across the field transect that could be correlated with apparent diversity as measured by DGGE. Soil features of site A that differed noticeably from those of the other sites included decreased pH, greater EC, and higher nitrate and ammonia concentrations. The source of ammonia is probably the hydrothermal steam permeating the soil at this site, since ammonia is a common feature in many geothermal features in Yellowstone National Park (11). Higher nitrate concentrations observed in this soil may be the result of biological nitrification of this ammonia, although this was not verified by biochemical measurements. The site A EC was considerably lower than that of seawater (51,000 μS/cm) and thus would not be expected to have profound selective effects. Furthermore, the pH of site B, which, like site A, was impacted by heat and showed reduced diversity in its DGGE profile, was almost identical to that of the cooler sites. In sum, the comparison of soil chemical properties across the field site leads to the conclusion that observed changes in microbial diversity across the landscape were most probably due to the underlying geothermal event.

DGGE analysis was used to semiquantitatively assess the temperature effect; it showed a very obvious change in soil community diversity across the thermal continuum (Fig. 3). This was the case for both the archaeal and bacterial communities and is consistent with the results from the intentionally simple agar plate culture experiment. The heat-impacted area had at least a 100-fold-greater number of cultivatable thermophilic bacteria (as colony counts) than did the nonimpacted soil. Thermophiles are probably distributed throughout this region and are subsisting at low levels or perhaps as spores in low-temperature environments, "awaiting" favorable conditions for growth. The results of the soil microcosm experiment added further support to this conclusion. Sequences retrieved from an originally nonthermal soil that was incubated at 50°C included near relatives from Yellowstone thermal soils (Table 2). These close relatives included a culture isolate, a clone library sequence, and a sequence from a DGGE profile, three different methods by which sequences were retrieved from the heat-impacted Ragged Hills soil. Five of the clones belonged to the genus *Alicyclobacillus*, members of which we have found in thermal areas throughout Yellowstone (unpublished data). All of these presumed thermophilic species necessarily had to be present at the start of the experiment in the nonthermal soil that was used as the starting material. Taken together, the results of the culture-based and molecular methods suggest that the environment had selected for these thermophilic species in the heat-impacted area, where they are now flourishing.

Because predicting the occurrence of dramatic environmental changes such as temperature shifts is nearly impossible, it is difficult to assess the initial community structure of the field site soils, although the nearby soil unimpacted by the heating event would provide the best approximation. Furthermore, there was no way to evaluate the successional stage of the thermally impacted soil community at the time the field study began. The soil microcosm experiment was an attempt to address these two issues in a more controlled laboratory setting (Fig. 6 and 7). In this experiment, each treatment started with the same initial soil community (well-homogenized soil sample), with temperature being the only variable. The result was that the community structure of a thermally impacted soil changed dramatically within 1 week and then appeared to stabilize into a new climax community within a period of 2 to 3 weeks. The rapidity with which such a successional community shift was observed makes it likely that our initial field samples, which were collected roughly 4 months after the heating event, represented those of a new climax community. In addition, the results of the microcosm experiment again provided evidence that viable thermophilic and thermotolerant bacteria subsist in nonthermal soils as well as thermal soils throughout the Yellowstone National Park landscape. Therefore, growth of thermophiles and thermotolerant species following a geothermal heating of soils in situ does not require (but may include) transport of these species from other thermal locations.

DGGE profiles from both the field transect (site B) and the soil microcosm experiment (soil taken from site F) demonstrated the selective pressure of temperature in significantly reducing the diversity of a soil community. Temperature effects have also been documented to exert selective effects in self-heating compost environments (5), although reduction in apparent diversity, as was observed in the present study, is not

always the case (24). In both the field site and the microcosm soils, thermophile populations seem to have flourished as a result of the heating, with sequencing results of bands derived from these profiles identifying representative species from different bacterial divisions. Although this experiment was conducted in a manner designed to simulate field conditions as closely as possible, the system was sufficiently perturbed that the increased temperature under laboratory conditions selected for different species from those observed in the field samples. Transfer of soil from the field to the laboratory, by whatever fashion, risks changing major soil structural features that contribute to defining the overall microbial community diversity and function. One example of how this could influence the present study would be in situ soil pore system continuity, which would affect soil moisture regimens and hence thermal conductivity at the system level and thus would contribute to differences between initial soil populations (prior to thermal disturbance). Another important example would be the naturally occurring redox gradients established by water films in conjunction with aggregate size and distribution. However, the fact that the soil was taken at a dissimilar time and slightly different site may also be responsible for the discrepancy.

Diversity has been examined in many types of presumed climax microbial communities in soils from many regions of the Earth, including Siberian tundra (31), grasslands of The Netherlands (9), Amazonian rainforest soils (4), U.S. agricultural soils (3), and arid soils of the southwestern United States (8, 14). Other reports have described the impact(s) of perturbations such as addition of methane or nitrogen (22), seasonal differences in agricultural soils (27), and differences between improved and unimproved pastures (16). A terminal RFLP examination of vertically distributed bacterial populations in rice paddy cores also showed significant changes in species composition along an oxygen gradient (15). In such studies, however, the ability to focus on a single environmental variable without soil disturbance or without complications arising from major changes in the prevailing chemistry (e.g., variation in Fe and S species and bioavailability due to redox gradients) can be very difficult.

While the potential for PCR- and DGGE-associated biases (21, 29) in the present study are clearly noted and acknowledged, we draw attention to the nearly identical DGGE profiles obtained from the DNA and RNA template populations. Metabolically active cells are engaged in ribosome synthesis proportional to growth (30), and thus diversity characterizations or estimates based on phylogenetic analysis of clones derived from RT-PCR directly from RNA templates might be expected to offer a closer link to the metabolically active fraction of any microbial community. Because of temporal fluctuations in environmental conditions (e.g., seasonal changes), it might be predicted that for any particular soil environment the metabolically active fraction is a subset of the total and that nearly complete agreement between DNA-based and RNA-based comparisons would be the exception and not the rule. However, in previous work with hot spring microbial mats, we have also encountered similar RNA- and DNA-based DGGE profiles (20). Also, in a recent study by Nogales et al. (19), a comparison of 16S rRNA- and rDNA-based clone libraries from a polychlorinated biphenyl-contaminated soil revealed that the distribution frequency of clones among phylogenetic groups was very similar between the two libraries. Similarly, in our study, DGGE profiles derived from PCR of DNA templates and RT-PCR of RNA templates were nearly identical (Fig. 4 and 7). Such close similarity between DNA- and RNA-based estimates might imply that the total population and the metabolically active fraction are one and the same. However, some caution is required in the interpretation of these results since even metabolically quiescent cells (and spores) retain at least a portion of their RNA complement, and thus it may be argued that RT-PCR of RNA templates would still detect populations that do not necessarily contribute significantly to the overall community biogeochemical activity.

The question of RNA and DNA turnover rates was revisited in the soil microcosm experiment. In this experiment, temporal changes in DGGE community profiles based on either RNA or DNA were compared. At the start of the experiment, the profiles were nearly identical, but 1 week following the temperature treatment they were quite different (Fig. 7). Interestingly, the community profiles were nearly identical to each other again after 4 weeks, although they were very different from the starting-community profiles. This was not an unexpected observation with this particular soil, given the DGGE profiles obtained from field samples, and it would be reasonable to assume that the elevated temperatures not only would select for thermophiles but also would kill temperature-sensitive bacteria, leaving behind carcasses that would supply significant fodder for existing heterotrophic thermophiles.

Because there was a very close correspondence between DGGE profiles of field transect samples based on rDNA and rRNA and because DNA is easier to work with than RNA, we chose to use DNA for clone library construction. A striking feature of the Ragged Hills clone library is the predominance of clones belonging to the *Acidobacterium* group. Of the 39 partially sequenced 16S rDNA clones, 51% had closest relatives (89 to 98% similarity) belonging to this group. The *Acidobacterium* group is a relatively newly recognized division within the *Bacteria*, with representatives from widely differing habitats, including hot springs, soils, acid mine drainage, and oceans (12). The majority of *Acidobacterium* sequences in the databases belong to soil bacteria, and the numbers are growing rapidly as more soil environments are surveyed. Even though the *Acidobacterium* group now comprises a relatively large division, little is known about their physiology because there are only three cultured representatives; *Acidobacterium capsulatum*, *Holophaga foetida*, and *Geothrix fermentans.* Based on the diversity of habitats and the depth of branching of sequences in phylogenetic analyses of acidobacterial environmental clones, it is likely that this group may eventually be shown to be as metabolically diverse as the *Proteobacteria* (12). Sequence similarity and phylogenetic placement show that our clones may include representatives of at least three of eight monophyletic subdivisions identified by Hugenholtz et al. (12). Clones 46A and 72A belong to group 1, and clones 2B and 5A belong to groups 2 and 3, respectively, with 100% bootstrap support. Considering the diversity and high proportion of acidobacteria in our clone library, this group might be considered to play an important, but as yet unknown, ecological role in the Ragged Hills soil system.

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materials have shown a rather limited broad-sense phylogenetic distribution of 16S rDNA clones (7, 24). By contrast, although the majority of clones from our soil clone library belonged to the *Acidobacterium* and *Planctomyces* groups, we did recover sequences with phylogenetic placement throughout the *Bacteria* domain. In this respect, the Ragged Hills thermal soil is similar to soils from many other temperate environments and locations.

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