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ORIGINAL PAPER

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Microbial diversity at 83°C in Calcite Springs, Yellowstone National Park: another environment where the *Aquificales* and "Korarchaeota" coexist

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Abstract The use of molecular phylogenetic approaches in microbial ecology has revolutionized our view of microbial diversity at high temperatures and led to the proposal of a new kingdom within the Archaea, namely, the "Korarchaeota." We report here the occurrence of another member of this archaeal group and a deeply rooted bacterial sequence from a thermal spring in Yellowstone National Park (USA). The DNA of a mixed community growing at 83°C, pH 7.6, was extracted and the small subunit ribosomal RNA gene (16S rDNA) sequences were obtained using the polymerase chain reaction. The products were cloned and five different phylogenetic types ("phylotypes") were identified: four archaeal phylotypes, designated pBA1, pBA2, pBA3, and pBA5, and only one bacterial phylotype, designated pBB. pBA5 is very closely related to the korarchaeotal phylotype, pJP27, from Obsidian Pool in Yellowstone National Park. The pBB phylotype is a lineage within the *Aquificales* and, based on 16S rRNA sequence, is different enough from the members of the *Aquificales* to constitute a different genus. In situ hybridization with bacterial-specific and *Aquificales*-specific fluorescent oligonucleotide probes indicated the bacterial population dominated the community and most likely contributed significantly to biogeochemical cycling within the community.

Key words Archaea · Korarchaeota · *Aquificales* · Hyperthermophiles · 16S rRNA

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Introduction

Despite the significant advances in the discovery and study of hyperthermophiles, the ecology of high-temperature microbial communities remains poorly studied (Baross and Deming 1996). However, the development of molecular techniques based on small subunit ribosomal RNA (16S rRNA) sequences has provided an approach for describing microbial diversity (for reviews, see Pace et al. 1986; Ward et al. 1992; Amann et al. 1995) in the environment without the need to grow the organisms. These assessments of microbial diversity provide a framework for ecological studies.

One of the most accessible and pristine thermal areas in the world for microbial ecological research is protected in Yellowstone National Park, USA. Studies have included enrichments and limited activity measurements (Brock and Darland 1970; Brock 1978; Slobodkin et al. 1997), although extensive ecological characterization of thermophilic (40°–60°C) and mesophilic communities has been conducted (Pierson and Olson 1989; Madigan et al. 1989; Ward et al. 1990, 1992). However, it has only been with the recent application of molecular tools in microbial ecology that many of the previously unidentified and as yet unculturable hyperthermophiles growing in these thermal springs have been identified. Previous studies of hyperthermophilic communities in Yellowstone National Park using the rRNA-based approach have identified the dominant member of a previously uncharacterized community (Reysenbach et al. 1994) and revealed the existence of a plethora of novel Archaea (Barns et al. 1994) and Bacteria (Hugenholtz et al. 1998) at high temperatures. The former study identified the "pink filaments" of Octopus Spring as a member of the *Aquificales*, the thermophilic hydrogen oxidizers (Kawasumi et al. 1984; Kristjansson et al. 1985; Huber et al. 1992; Shima and Suzuki 1993) that obtain their energy re-

quirements from the “knallgas” reaction (Aragno 1990). Based on the sequence and physiological affiliation of the pink filaments with the *Aquificales*, these previously uncultivated organisms have now been grown in the laboratory (Huber et al. 1998). Additional 16S rRNA sequences of the *Aquificales* were obtained from another thermal spring in Yellowstone, Obsidian Pool (Hugenholtz et al. 1998) and from thermal springs in Japan (Yamamoto et al. 1998). In Obsidian Pool, the communities are predominantly bacterial although many novel lineages within the Crenarchaeota have been identified (Barns et al. 1994), in particular, the lineage and newly proposed kingdom, the “Korarchaeota” (Barns et al. 1996). In this study we analyzed the microbial diversity of the black filamentous community associated with thermal springs at Calcite Springs in Yellowstone National Park. We report here the detection of another member of the “Korarchaeota” and show that a member of the *Aquificales* dominates this hyperthermophilic community. We propose that these communities may be pivotal in the study of the early evolution of life.

Materials and methods

Sampling site and collection

Samples of the black filamentous biomass associated with thermal features at Calcite Springs at Yellowstone National Park, WY, USA (44°54.291′ N, 110°24.242′ W) were collected aseptically with tweezers and frozen in liquid nitrogen. The temperature at the sampling point was 83°C and the pH was 7.6. The thermal springs are shallow (1–5 cm deep) streams with a source temperature of 93°C and a temperature gradient along the streams from 93° to approximately 50°C, at which stage the springs enter the Yellowstone River. The black filamentous organisms appear to dominate the streams between 60° and 84°C, although visual evidence of occasional filamentous organisms have been noted up to 89°C.

Microscopy

The samples were viewed by phase-contrast microscopy and fluorescent microscopy of 4′,6′-diamidino-2-phenylindole (DAPI) stained preparations (Porter and Feig 1980) using an Olympus BX60 microscope. In situ hybridization analysis of ethanol-fixed (70% v/v ethanol in phosphate-buffered saline) samples was performed as previously described (DeLong et al. 1989; Reysenbach et al. 1994). Fluorescein-labeled bacterial-specific (5′-ACCGCT TGTGCGGGCCC-3′) and archaeal-specific (5′-GTGCTC CCCCGCCAATTCCT-3′) (Burggraf et al. 1994) oligonucleotide probes were used to determine the relative abundance of Archaea and Bacteria. The presence of a phylotype related to the *Aquificales* was determined using two different probes designed for the *Aquificales*, S-O-Aqui-1459-a-A-16 (5′-ATCGTCCTCTGCCTCC-3′) and S-O-Hydr-0540-a-A-19 (5′-TCGCGCAAGCTCGGGA CC-3′) (Harmsen et al. 1997). The in situ hybridizations were viewed with an Olympus BX60 microscope.

DNA extraction and 16S rRNA gene amplification

The DNA was extracted from the samples using a modified microwave and detergent (sodium dodecyl sulfate, SDS) lysis procedure (Bollet et al. 1991) as described previously (Reysenbach et al. 1994). The sample was suspended and incubated at 65°C for 30 min in TE (10 mM Tris-HCl, pH 8; 1 mM EDTA) containing 3.3% SDS. The sample was then heated in a microwave oven for four bursts (900 W) of 30 s each. The DNA was extracted three times from the suspension with equal volumes of phenol : chloroform : isoamyl alcohol (50 : 49 : 1), precipitated with 2 volumes of ethanol and 0.3 M sodium acetate, washed in 70% ethanol, and resuspended in TE.

The community 16S rRNA genes (16S rDNA) were amplified by PCR using two different forward primers to amplify specifically the bacterial (8F: 5′-AGAGTTTGAT-CCTGGCTCAG-3′) or archaeal (4F: 5′-TCCGGTTGA-TCCTGCCRG-3′) 16S rDNA. The reverse primer used for both amplifications corresponded to the complement of positions 1510 to 1492 of *Escherichia coli* 16S rRNA (1492RPL: 5′-GGCTCGAGCGGCCGCCCGGGTTACC-TTGTTACGACTT-3′). The PL refers to a 5′-polylinker tail containing restriction endonuclease sites to facilitate cloning. Amplification of the 16S rRNA genes was accomplished as described previously (Reysenbach et al. 1994; Reysenbach and Pace 1995). Each reaction (100 μl) contained DNA (20–100 ng), 10 μl 10× reaction buffer (Reysenbach and Pace 1995); 5 μl Nonidet P-40 (or Igepal; Sigma, St. Louis, MO, USA); 5% (w/v) acetamide, 0.2 mM (each) dATP, dGTP, dTTP, dCTP; 0.5 μg of the forward and reverse primers; and 1 U of *Taq* polymerase. The reactions were incubated in a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) for 4 min at 94°C, and then for 30 cycles of 1 min and 30 s at 92°C, 1 min and 30 s at 50°C, and 2 min at 72°C. The last step was extended by 5 s after each cycle. The PCR products were extracted with an equal volume of phenol : chloroform (50 : 50), precipitated with ethanol, and resuspended in TE. The PCR products were cut with *Eco*RV and *Not*I endonucleases and cloned into pBluescript KS– (Stratagene, La Jolla, CA, USA). Sixty clones containing inserts were screened using a rapid plasmid screening procedure (Sekar 1987), and unique clones were identified by single-nucleotide (ddT-terminated) sequencing patterns.

Both strands of the 16S rDNA of representative clones were manually sequenced using the dideoxy chain termination method, and sequences were confirmed by cycle sequencing and using an automated DNA sequencer Model 377 (Applied Biosystems, Foster City, CA, USA). A suite of both reverse and forward 16S rDNA-specific primers was used (Reysenbach and Pace 1995), and approximately 1466 nucleotides were sequenced. All sequences were submitted to Genbank and assigned by AF 113542 and AF176344–AF176347.

Phylogenetic analysis

Sequences were manually aligned using the GDE multiple sequence editor with a subset of 16S rRNA sequences from the ribosomal database project (RDP; Maidak et al. 1999) and recent 16S rRNA submissions to GenBank. Alignments were based on secondary structural features, and the presence of chimeric molecules was examined using CHECK_CHIMERA (RDP; Maidak et al. 1999). Only homologous and evolutionarily conserved nucleotides were used; approximately 1399 nucleotides were used in the analysis. The phylogenetic trees were constructed both with evolutionary distance matrices using the algorithm of DeSoete (DeSoete 1983) and by maximum-likelihood analysis, using the program fastDNAml (version 1.0, distributed by RDP; Maidak et al. 1999; Felsenstein 1981). Boostrap values were calculated for the maximum-likelihood trees by using 100 replicate trees and random sequence addition.

The GenBank accession numbers of the 16S rRNA sequences of the organisms used in this analysis are as follows: *Aquifex pyrophilus* [M83548], *Bacillus subtilis* [X60646], *Calderobacterium hydrogenophilum* [Z30242], *Chlorobium vibrioforme* [M62791, M27804], *Cytophaga hutchinsonii* [M58768], *Deinococcus radiodurans* [M21413], *Desulfurococcus mobilis* [M36474], EM17 [U05661], *Escherichia coli* [J01859], *Fervidobacterium islandicum* [M59176], GANI4 [AB005736], *Giardia* [AF006677], *Hydrogenobacter thermophilus* strain TK.6 [Z30214], *Hydrogenobacter thermophilus* strain *T3* [Z30189], *Methanococcus jannaschi* [M59126], *Methanococcus vannielii* [M36507], NAK9 [AB005737], pBA1 [AF176345], pBA2 [AF176346], pBA3 [AF176344], pBA5 [AF17647], PBB [AF113542], pJP 27 [L25852], pJP 78 [L25303], pJP 81 [L25304], pSL 60 [U63364 (5′end) and U63365 (3′end)], pSL 91 [U63360 (5′end) and U63361 (3′end)], *Pyrobaculum islandicum* [L07511], *Pyrodictium occultum* [M21087], *Streptomyces coelicolor* [Y00411, M35377], *Sulfolobus solfataricus* [X03235], *Thermococcus celer* [M21529], *Thermodesulfotobacterium commune* [L10662], *Thermofilum pendens* [X14835], *Thermoproteus tenax* [M35966], *Thermotoga maritima* [M21774], and *Thermus ruber* [L09672].

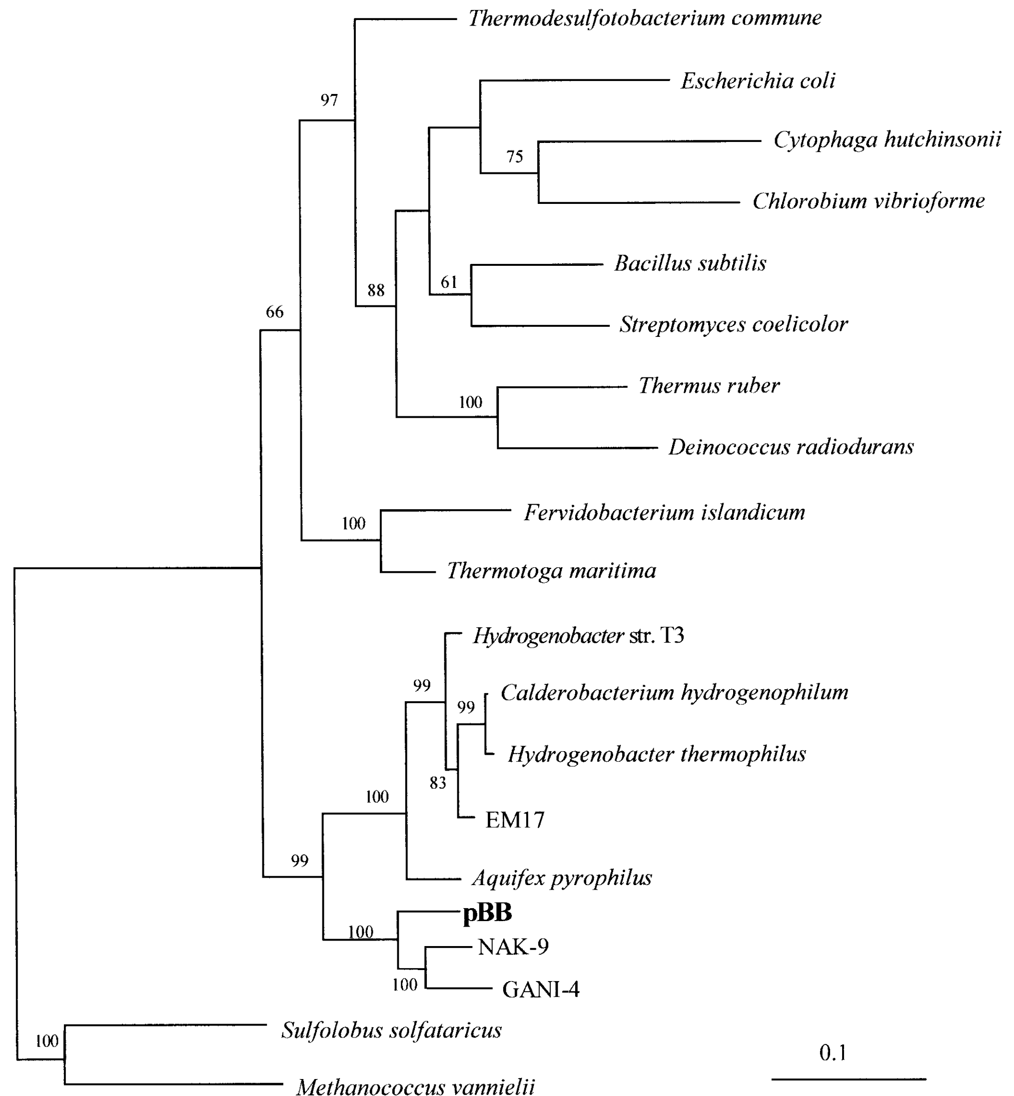

Fig. 1. Maximum-likelihood phylogenetic tree of bacterial 16S rDNA gene clone (pBB) obtained from an 83°C sample at Calcite Springs, Yellowstone National Park. The *scale bar* represents the number of fixed mutations per nucleotide position. The *numbers* at the branch nodes are bootstrap values based on 100 bootstrap resamplings

Results and discussion

Based on the molecular phylogenetic approach used in this study, the microbial community associated with the thermal springs at Calcite Springs appears to be limited in diversity (this study, at 83°C; from 93° to 60°C, unpublished results). A single bacterial clone type (Fig. 1; "phylotype" pBB) was detected from the community at 83°C, whereas 4 unique archaeal phylotypes were detected (Fig. 2; pBA1, pBA2, pBA3, pBA5). A problem with the described cloning approach is that 16S rDNA molecules may have had internal *Eco*RV and *Not*I sites resulting in clones with inserts of reduced size. However, in our experiments, all clones had inserts of the correct full-length size (about 1.5 kb). Although 30 bacterial clones were screened, phylotypes that differed by less than 0.1% were considered variants of the same phylotype. This clustering of variants or strains appears to be a common theme with molecular phylogenetic analyses of microbial communities (Angert et al. 1993; Moore et al. 1998) and may reflect microheterogeneity among different populations.

The limited diversity of the Calcite Springs community at 83°C is not surprising, because only organisms capable of attaching to each other and to the rocks and sediment within the stream can thrive in the stream flow. This is similar to the limited diversity observed in the stream at Octopus Spring in Yellowstone National Park (Reysenbach et al. 1994). In contrast, the highly mixed nonstream thermal environment of Obsidian Pool may support a much greater diversity of microorganisms because the organisms are not continually being washed downstream and away from optimal growth conditions.

The 83°C community at Calcite was dominated by thin (approximately 0.5 μm in diameter) rods that vary from 2 to 8 μm in length. The rods appear to be held together by a black iron mineral precipitate (Reysenbach and Beveridge, unpublished data) that is associated with the sample. This precipitate made viewing the organisms difficult by phase-contrast microscopy. The filamentous appearance of the communities may result from the clumping of the cells into filamentous masses of precipitate and cells (Fig. 3a). In situ hybridization with bacterial- and *Aquificales*-specific oligonucleotide probes revealed that the majority (>95%) of the community was bacterial, and presumably most of the bacterial types were the pBB phylotype (Fig. 3), although a pBB-specific probe must be designed to prove this definitively. Therefore, it is likely that these bacterial phylotypes are important members of the community. The archaeal probe cross-reacted with the filaments, which is consistent with this probe cross-reacting with *Aquifex pyrophilus* (Burggraf et al. 1994).

The bacterial phylotype, pBB, represents a separate lineage within the *Aquificales* (see Fig. 1). This phylotype has no known close relatives in culture, and the 16S rRNA sequence is only 77.7% and 80.5% similar to *Aquifex pyrophilus* and *Hydrogenobacter thermophilus*, respectively. The pBB sequence is very similar (98%) to sequences obtained from the bacterial community in Obsidian Pool, Yellowstone National Park (OPB13; Hugenholtz

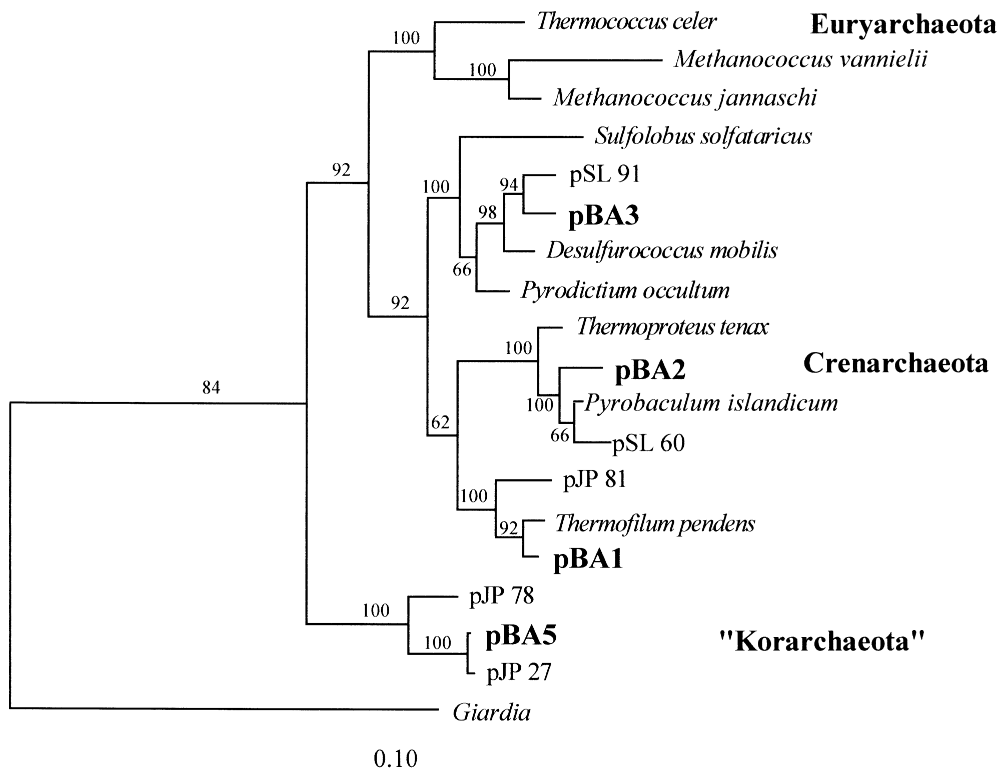

Fig. 2. Phylogenetic analysis of archaeal 16S rDNA gene clones (pBA1, -2, -3, -5) obtained from an 83°C sample at Calcite Springs, Yellowstone National Park. The tree was determined using maximum-likelihood analysis. The eukaryal sequence of *Giardia* was used as the outgroup in the analysis. The *scale bar* represents the number of fixed mutations per nucleotide position. The *numbers* at the branch nodes are bootstrap values based on 100 bootstrap resamplings

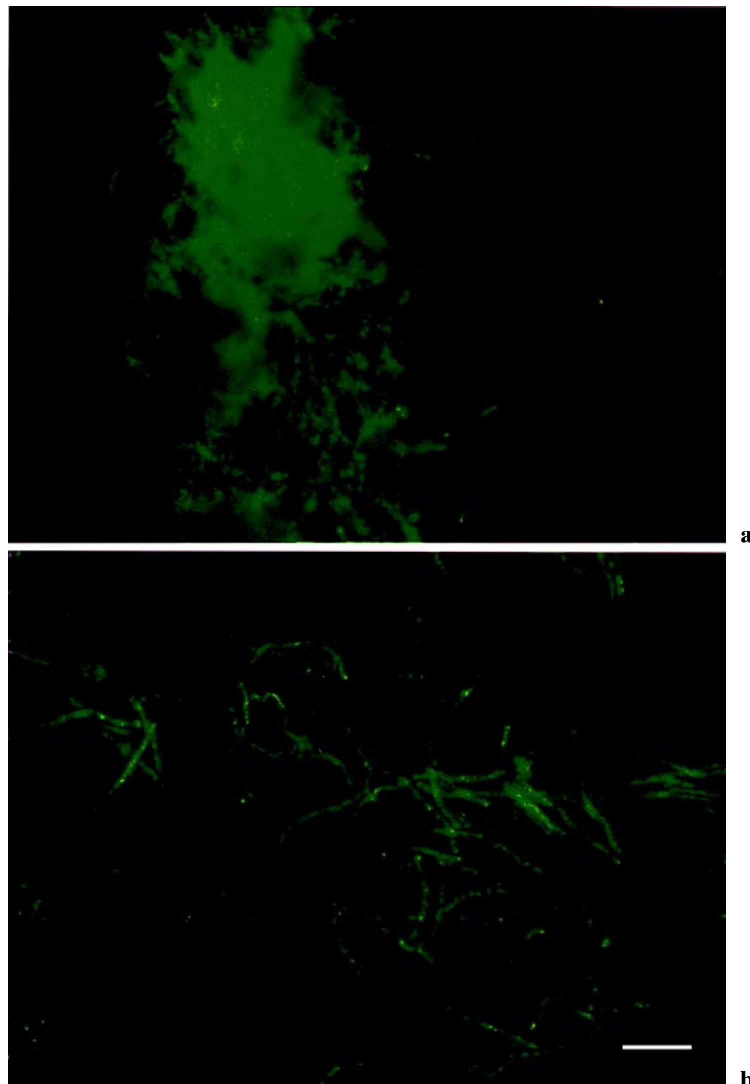

Fig. 3a,b. In situ hybridization of the black filamentous community with a fluorescein-labeled oligonucleotide specific for the *Aquificales* 16S rRNA. **a** Community "filament." **b** Individual bacterial cells. *Bar* 5 μm

et al. 1998) and sequences (95%–99% similar in 16S rRNA sequence; Graber et al., submitted) obtained from a stream that flows into Obsidian Pool and from thermal springs in Furnas, Azores (Reysenbach, unpublished results). Additionally, the sulfur turf 16S rRNA sequences from Japanese thermal springs also group together with this lineage (Yamamoto et al. 1998). Furthermore, this novel group is dissimilar enough from the other members of the *Aquificales* (>20%) to be regarded as a different genus and perhaps a different family.

Four different archaeal phylotypes were identified in the community (Fig. 2); 40% (12 of 30) of the clones were pBA5, a very close relative (99.6% 16S rRNA sequence similarity) of the proposed korarchaeotal phylotype, pJP27 (Barns et al. 1996). pBA5 does not share the same intradomain signature at position 503:542 with pJP27. Instead of the pJP27 G:C pair in this position, pBA5 has an A:U pair. This position change was confirmed by both manual and automated sequencing in 2 independent clones. pBA1 was the next dominant phylotype (26%, 8 clones) and most closely related to *Thermofilum pendens* and the partial Obsidian Pool phylotypes pJP6 and pJP81. Clones pBA2 and pBA3 each represented 17% of the clones sequenced and are most closely related to *Pyrobaculum islandicum* (and partial phylotypes pSL60, pJP8) and *Desulfococcus mobilis* (and partial phylotypes pSL91, pJP74), respectively. No euryarchaeotal clones were obtained in this analysis. The Crenarchaeota were originally distinguished from the Euryarchaeota by their preference for high-temperature acidic conditions. However, the discovery of Crenachaeota in temperate and Antarctic marine environments and in soils (DeLong 1992; DeLong et al. 1994; Hershberger et al. 1996), and the presence of the neutrophilic Crenarchaea described here, demonstrates that this kingdom has greater physiological diversity than previously described. Additionally, as pBA1 and pBA2 are both related to known hydrogen-utilizing isolates, the role of hydrogen oxidation in these ecosystems cannot be underestimated. Furthermore, it is interesting to note that, as pBA2 is closely related to the microaerophilic organism

Pyrobaculum islandicum, it is possible that, like pBB, pBA2 may have a hydrogen-utilizing microaerophilic metabolism.

The finding that the members of the Korarchaeota (pJP27, pBA5) and the new lineage within the *Aquificales* (OPB13, pBB) occur in both Obsidian Pool and Calcite Springs suggests that these organisms have overlapping niches and that some commonality may exist in their growth requirements, such as pH near neutrality, iron-rich environments, and overlapping temperatures for growth. Detailed geochemical measurements will further clarify these growth conditions. Additionally, enrichments for the "Korarchaeota" have been successful, and pJP27 from Obsidian Pool has been obtained in a mixed culture (Burggraf et al. 1997). This phylotype represented a very small portion of the total Obsidian Pool enrichment (Burggraf et al. 1997); however, as the Calcite Springs community is much less diverse than that of Obsidian Pool, chances of obtaining a larger population of the "Korarchaeota" may be greater if a sample from Calcite Springs is used as an inoculum. Furthermore, because pBB and pBA5 share overlapping niches, we are currently attempting to obtain these in coculture.

Although the archaeal diversity in this study is greater than the bacterial diversity, based on in situ hybridization results, the archaeal community appears to form only a small part of the total biomass of the community. It is therefore tempting to speculate that the bacterial member (pBB) drives the prevalent biogeochemical processes in this ecosystem, with the archaeal members playing minor roles in biogeochemical cycling. The prevalence of filamentous bacterial hyperthermophiles such as the *Aquificales* in high-temperature ecosystems (Reysenbach et al. 1994; Reysenbach, unpublished data) points to the possible importance of these organisms in driving the biogeochemical cycles in these ecosystems. Furthermore, because these geothermal environments are habitats for some of the most deeply rooting organisms within the rooted universal tree of life, their physiology may provide insight into the physiology and environmental requirements that were needed for the evolution of early life on Earth.

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