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Effects of Abiotic Factors on the Phylogenetic Diversity of Bacterial Communities in Acidic Thermal Springs[▽]

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Acidic thermal springs offer ideal environments for studying processes underlying extremophile microbial diversity. We used a carefully designed comparative analysis of acidic thermal springs in Yellowstone National Park to determine how abiotic factors (chemistry and temperature) shape acidophile microbial communities. Small-subunit rRNA gene sequences were PCR amplified, cloned, and sequenced, by using evolutionarily conserved bacterium-specific primers, directly from environmental DNA extracted from Amphitheater Springs and Roaring Mountain sediment samples. Energy-dispersive X-ray spectroscopy, X-ray diffraction, and colorimetric assays were used to analyze sediment chemistry, while an optical emission spectrometer was used to evaluate water chemistry and electronic probes were used to measure the pH, temperature, and E_h of the spring waters. Phylogenetic-statistical analyses found exceptionally strong correlations between bacterial community composition and sediment mineral chemistry, followed by weaker but significant correlations with temperature gradients. For example, sulfur-rich sediment samples contained a high diversity of uncultured organisms related to *Hydrogenobaculum* spp., while iron-rich sediments were dominated by uncultured organisms related to a diverse array of gram-positive iron oxidizers. A detailed analysis of redox chemistry indicated that the available energy sources and electron acceptors were sufficient to support the metabolic potential of *Hydrogenobaculum* spp. and iron oxidizers, respectively. Principal-component analysis found that two factors explained 95% of the genetic diversity, with most of the variance attributable to mineral chemistry and a smaller fraction attributable to temperature.

Molecular studies of geothermal ecosystems have provided many illuminating discoveries of microbial diversity (3, 23, 36, 45). Culturing methods and molecular studies have uncovered a remarkable diversity of both bacteria and archaea in hot springs worldwide. Extremophile microbial communities are particularly interesting because their habitats may resemble anoxic volcanic habitats thought to have existed on early Earth (17, 36). Indeed, many of the bacterial lineages identified from hot springs appear to be related to lineages close to the root of the bacterial tree (36). Hot springs have been suggested as model systems for extraterrestrial life (8, 17), and organisms isolated from hot-spring environments (e.g., *Thermus aquaticus*) have also proven to be valuable in biotechnology and research applications (7).

Despite decades of research, we still understand relatively little about the ecological factors and evolutionary processes dictating extremophile community diversity. This knowledge gap can largely be explained by the fact that molecular methods for exploring microbial diversity are recent in origin (1, 15, 22, 36, 44, 48, 49). Prior to the invention of PCR-based cloning techniques for exploring microbial diversity, culturing methods were the primary means for identifying new microorganisms. However, the utilization of molecular methods based on rRNA gene sequences have revealed a vast diversity of previously uncultured microbes in hot springs and many other environments (1). The small size of microbes, their vast numbers, and the difficulty of applying species concepts to microbes have also been impediments to statistical analyses (4, 24, 31). However, the recent development of phylogenetically based statistical approaches for analyzing environmental sequence data has greatly enhanced the ability to study microbial community diversity (4, 31).

To better understand how abiotic factors affect extremophile diversity, we designed a comparative 16S rRNA gene PCR-based molecular study of acidic hydrothermal springs at two locations in Yellowstone National Park (YNP), Amphitheater Springs (AS), and Roaring Mountain (RM). The combination of high temperature (~75°C) and low pH (<2) in these sulfur- and iron-dominated springs appears to make them inhospitable, yet acidic thermal springs teem with life (2, 23). In our study, we considered the environmental parameters of temperature and sediment composition prevailing in these springs. Evidence from the scientific literature indicates that each of these factors appears to affect microbial diversity to some degree.

In this study, we tested two main hypotheses concerning the respective influence of abiotic factors on microbial community diversity. The first hypothesis (H1) tested was that substrate mineral chemistry plays the dominant role in shaping the organismal composition of acidic thermal spring communities (10, 14, 33, 36, 37). Underlying chemistry of microbial environments appears to be critical for determining community composition, especially in acid hot-spring environments, where chemolithotrophs are reported to be the primary producers

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(16, 25). Given this hypothesis, we tested the following predictions: (i) samples from springs with the most similar chemistries will have the greatest overlap in bacterial diversity, (ii) this pattern will persist despite differences in temperature or geographic distance, and (iii) most of the microbial sequence diversity in springs should be attributable to chemistry, which implies metabolic potential.

The second hypothesis (H2) tested was that temperature gradients substructure the microbial communities within springs (6, 32). It goes without saying that temperature affects microbial growth. What is less clear is whether the effects of temperature on microbial diversity can be detected in geothermal springs over gradients of 5 or 10°C. In a comparative study of the microbial-mat communities in hot springs, Skirnisdottir et al. showed that the communities at the most extreme temperatures had the lowest overall bacterial diversity (41). Another study by Chapelle et al. suggested that archaea predominate under more-extreme environmental conditions (8). If temperature gradients substructure communities within springs, we predict that (i) samples collected from the same spring at different temperatures will differ significantly in bacterial diversity and (ii) samples collected at the same temperature from different streams with similar chemistries should be more similar than samples from the same stream at different temperatures. Generally, we expect that temperature will explain some of the variation in bacterial diversity of acidic thermal-spring communities but less than chemistry.

MATERIALS AND METHODS

Study sites. AS is characterized by highly acidic soils and six flowing sulfur springs comparable in pH, temperature, and mineral chemistry (Fig. 1A and B). The AS is also in close geographic proximity (~2 km) to an acidic, flowing, iron-rich spring, RM, which permits diversity comparison of two iron-rich springs, AS and RM, with similar pHs and temperatures (Fig. 1A and C). The acidic thermal springs chosen for this study (Fig. 1) provide natural laboratories for determining the types of ecological processes that affect the composition of microbial communities. Maps of RM and AS have been included to indicate relative and local distances (Fig. 1D). One of the springs (AS103) in this area has both iron and sulfur deposits that allow for within-spring comparisons of bacterial diversity in different mineral sediments.

Figure 1A shows the four features of our design enabling our comparative study. First, all of the springs have the same approximate pH of <2. Second, temperature gradients along these springs were similar in the springs we examined. Third, independent springs with similar chemistries allow replication of the effects of chemistry on community diversity. Fourth, the mixed iron-and-sulfur chemistry of spring AS103 allows within-spring correlations of chemistry and community diversity.

Sample collection. Water and sediment samples were collected in the summer of 2004 in gamma-irradiated, sterile 50-ml polypropylene tubes by using propipettes and individually wrapped sterile 25-ml plastic pipettes. Figure 1A shows the number of stations sampled and their approximate locations. The samples were immediately frozen on site in liquid nitrogen. Samples for cell counts and phase-contrast microscopic analysis were collected in a similar fashion but stored in 4% paraformaldehyde at 4°C. Cells were stained with 4′,6′-diamidino-2-phenylindole (DAPI) and visualized with a Leitz M20 UV light epifluorescence microscope. Water samples and samples of visible deposits of iron and sulfur were collected in sterile, gamma-irradiated 50-ml polypropylene tubes. Iron and sulfur samples were obtained by scraping the deposits. All samples were preserved in liquid nitrogen on site until storage at −80°C in the laboratory.

Water and sediment chemistry. Water pH, E_h, and temperature were measured with a Lamotte pH plus direct meter. Temperature was measured with a maximum-recording thermometer. These measurements were made in both May 2004 and August 2004 to establish the constancy of the temperature and pH conditions over time. Elemental water analysis was performed with a Perkin-Elmer Optima 4300 DV inductively coupled plasma (ICP) optical emission spectrometer (Perkin-Elmer, Shelton, CT). Nutrient water analysis was done with a Lachat model 8000 flow injection analyzer (Lachat, Loveland, CO). Amorphous and crystalline Fe(III) oxides were extracted from the pool of substrate iron in a sediment sample (0.1 g) by reduction with 500 μl 0.1 M Na dithionite in the presence of 0.1 M Na citrate. The duration of the extraction was 24 h at 37°C, followed by centrifugation at 10,000 × *g* for 5 min. The supernatant was then assayed for total iron with 1,10-phenanthroline (19). For Fe(II) analysis of substrates, a 500-μl aliquot of 0.5 N HCl was added to the substrate (0.1 g) for 1 h at 23°C. HCl-extractable Fe(II) was then determined with the phenanthroline assay as described above. Controls showed that Fe(II) (as ferrous sulfate) was not oxidized by the extraction and HCl did not interfere with the assay.

For sediment analysis of springs, 100-mg samples were filtered onto a 13-mm-diameter 0.22-μm-pore-size Millipore membrane, washed with distilled water, and transferred while wet to a double-stick carbon conductive tab (Ted Pella, Redding, CA). Carbon-coated or Au-Pd-coated samples were analyzed by scanning electron microscopy (SEM) with a Hitachi 2700 operated at 20 kV with an Oxford Instruments X-ray microanalyzer with Inca software to perform energy-dispersive X-ray spectroscopy (EDS). Samples to be analyzed by X-ray diffraction (XRD) were washed with acetone, pulverized, transferred to a cleaned mineralogy slide, and dried. Analysis was completed in a Philips X'Pert MPD Pro Theta/Theta powder XRD system with the X'Pert modular software and JCPDS reference database and retrieval software (http://www.icdd.com/). Iron was also assayed by the 1,10-phenanthroline method (9).

DNA extraction, PCR, and cloning. All samples were adjusted to pH 8 with 5% sterile-filtered KOH (0.22 μm; Millipore) prior to DNA extraction. Total genomic DNA was extracted from all samples by means of an ultraclean soil DNA purification kit (MoBio, Solana Beach, CA) by following the manufacturer's instructions. Approximately 1 ml of the sample (water and sediment) was suspended with silicon beads for extraction on a vortexer for 30 min, allowing for complete lysis of cells. 16S rRNA gene sequences were amplified by PCR with universal bacterial primers 8F and 805R (1). The PCR conditions included an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 45 s, and extension at 72°C. This was followed by a final extension at 72°C for 20 min (27). One microliter (~5 ng) of DNA was amplified in 50 μl of reaction mixture for 35 cycles, which was the minimum number of cycles needed to get a sufficient PCR product. The PCR products amplified by the universal primer pair included a variable region of the 16S rRNA gene useful for phylogenetic analysis. The PCR products were purified with a commercially available spin column purification kit from the QIAGEN Corporation (Valencia CA) prior to cloning. Clone libraries were constructed with the commercial cloning kit pGEM (Promega, Madison, WI) according to the manufacturer's instructions. Transformants were first checked for inserts by PCR with M13 primers. Minipreps and sequencing, with the 8F primer, were performed at the San Diego State University MicroChemical Core Facility.

Data analysis. BLAST was used to determine if the resulting rRNA gene sequences were similar to those of characterized organisms. The ARB software package (http://www.arb-home.de), a program commonly used for databases of environmental rRNA gene sequences, was used to create rigorous, structure-based alignments of rRNA gene sequences (29). The FastGroup II analysis program (http://biome.sdsu.edu/fastgroup/fg_tools.htm) was used to edit cloned sequences and "dereplicate" sequences. Dereplication is the process of determining which sequences in the clone library are identical within a given percent similarity and grouping them together. The program was used to trim vector sequence along with any unwanted sequence. The rRNA gene sequences were trimmed from the 3′ end at the bacterial 534 conserved site found in all known bacterial sequences. Sequences with a percent sequence identity of greater than 97% were placed in the same dereplicated group. One sequence from each group was selected as a representative operational taxonomic unit (OTU).

Statistical analyses. We used FastGroup II to calculate the Shannon-Weiner index and coverage of each sample after dereplication. Each OTU was considered a separate species for these analyses. The Shannon-Weiner index (H) was used to summarize the diversity of an ecological community, represented by $H = -\Sigma p_i \ln p_i$, where p_i is the frequency of the ith species (31). Coverage (C) was calculated with the equation $C = 1 - n/N$, where n is the number of unique OTU sequences observed and N is the total number of OTUs (i.e., the sum of unique OTUs plus OTUs observed more than once) (43). This coverage value assessed the amount covered from what was sampled and may not necessarily reflect the full diversity of the organisms. The PAUP* phylogenetic program (47) was used to run the permutation tail probability test (P test), while the Arlequin program, a population genetics software package, was used to calculate the F statistic for the analysis of molecular variance (Φ_{ST}). Other studies have used Φ_{ST} to estimate the genetic overlap of microbial communities, and we use the same approach here (31, 51). The P test was used to determine whether there were

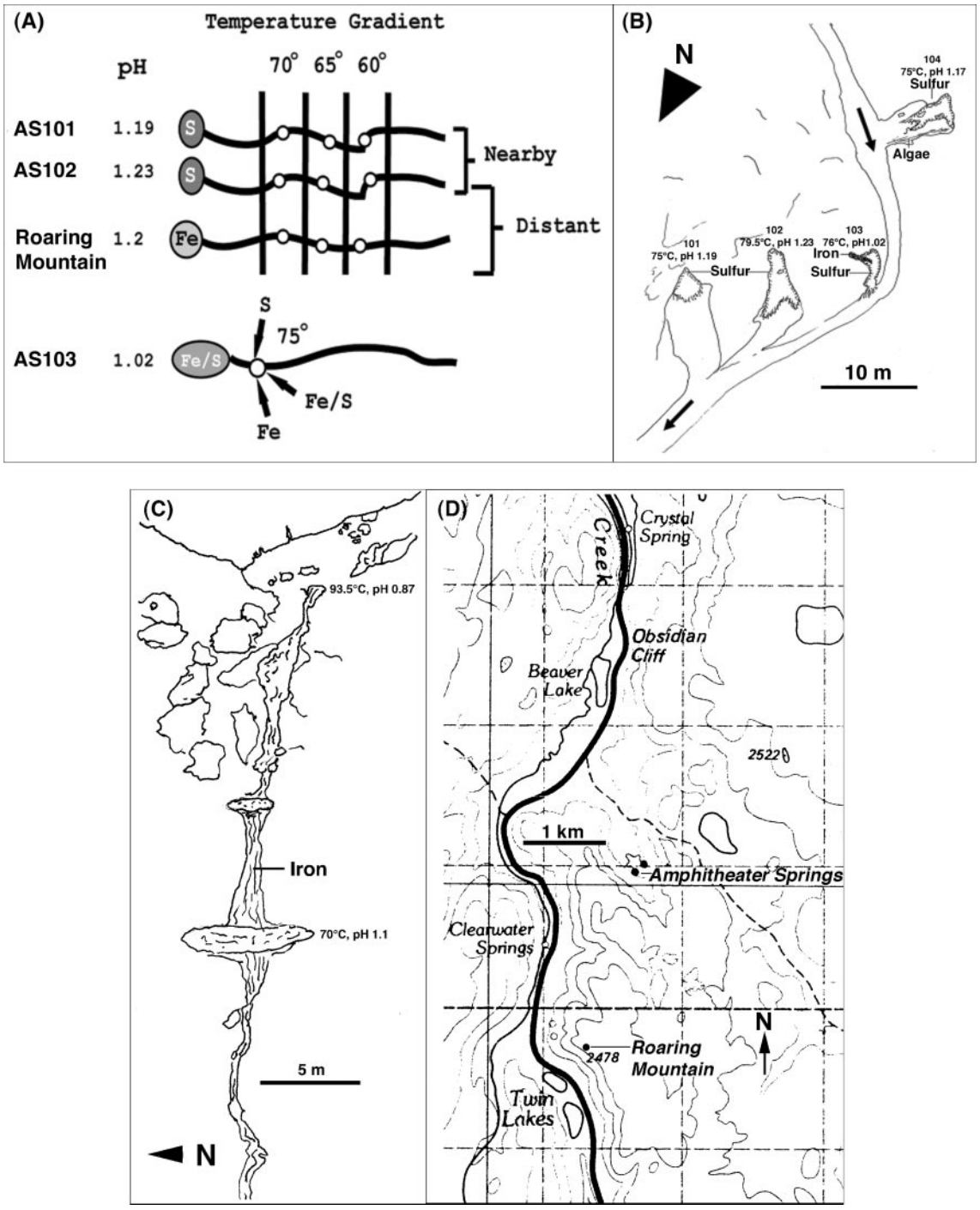

FIG. 1. (A) Study design diagram of the four springs. Ovals indicate the spring source (S = sulfur, Fe = iron-dominated spring), and open circles indicate sample collection points. (B) Flowing spring at RM. Origin and 70°C sample site (lower of two pools) are marked with temperature and pH. (C) Flowing springs at AS. (D) Contour map of AS and RM (1:100,000 scale) based on a 1983 U.S. Geological Survey 50-min interval map (Yellowstone National Park North, Wyoming-Montana).

correlations between environments and phylogeny (31). Φ_{ST} assesses the degree of differentiation between bacterial communities (31, 38) by comparing the genetic diversity within each community to the total genetic diversity of the communities combined. This is given by the equation $\Phi_{ST} = (\theta_T - \theta_W)/\theta_T$, where θ_T is the genetic diversity of all of the samples (all of the communities combined) and θ_W is the genetic diversity within each community (31).

This statistic is also a useful measurement of how similar two communities are in their organismal diversity. The Φ_{ST} analysis used the estimated distribution of data from clone libraries, which can be a potential problem if PCR bias overestimates or underestimates the diversity of particular bacterial species. However, our community diversity was quite high, suggesting that PCR bias did not have a particularly pronounced effect on our results. Statistical significance was evaluated by randomly assigning sequences to populations and calculating the Φ_{ST} for 1,000 permutations. The pairwise Φ_{ST} values between all samples were

reduced by principal-component analysis (PCA), allowing the genetic clustering of samples to be visualized in two dimensions. The Φ_{ST} values represent the dissimilarity between each pair of samples, so the set of pairwise Φ_{ST} values between a given sample and all other samples produces an *n*-dimensional vector that describes the relationship between it and the other samples. The factor through space that describes as much variation as possible is the principal coordinate. Factor analysis reduced the dimensionality to a manageable number of principal components, allowing simplified visualization and analysis. PCA has been used extensively to compare microbial communities with complex data sets such as terminal restriction fragment length polymorphism-denaturing gradient gel electrophoresis profiles, fatty acid profiles (52), and substrate use patterns (28). However, PCA has not previously been applied to Φ_{ST} values. This technique also allowed the main components of genetic variability to be regressed with respect to environmental variables (chemistry, temperature, and geography) by general linear model regressions. These analyses were carried out with SYSTAT, version 10.

Phylogenetic analysis. All phylogenetic analyses were performed with sequences that were aligned with full-length 16S rRNA gene sequences from GenBank (29). Tree visualization and initial phylogenetic identification were done with the tools available in ARB (29). Maximum-likelihood (ML), neighbor-joining (NJ), and maximum-parsimony (MP) criteria were used to determine the relationships among sequences (47). The ML and MP analyses included 100 heuristic random-addition sequence searches to find the highest-likelihood and most parsimonious trees, respectively. ML analyses used the HKY85 model of sequence evolution with estimated transition/transversion ratios and nucleotide frequencies. Bootstrap analyses were performed by ML, MP, and NJ. One thousand NJ bootstrap replicates were performed for each data set, while 100 bootstrap replicates were performed under the ML and MP criteria with 10 heuristic random-addition sequence searches per replicate.

Nucleotide sequence accession numbers. The sequences described in this report have been deposited in GenBank under accession numbers AY934906 to AY935164.

RESULTS

X-ray and chemical analyses. DAPI staining and SEM analysis of samples showed scattered cells from AS and RM samples including cells attached to sulfur at AS102 (Fig. 2). Mineral identification based on visualization of iron (red-rust) and sulfur (yellow) deposits was carried out by SEM. Representative spectra illustrating these chemically defined sites are shown in Fig. 3. Mineral coating of the substrate by silicon, iron, manganese, molybdenum, and other elements was examined in three different ways, i.e., (i) by analysis of solid mineral substrates by SEM (EDS), (ii) by XRD of acetone powders, and (iii) by wet colorimetric analysis of Fe. Table 1 summarizes the main results of these collective assays. In the first approach, EDS, the majority of scans consistently revealed major silicon and oxygen peaks. Sulfur was not seen as a major peak in multiple scans of the reddish Fe substrate of AS103, but it was detected in about 30% of the spectra as a minor constituent. The second method, XRD, also examined the reddish Fe substrate and revealed individual lines for Fe, Mn, and Mo, but other lines expected for Fe and/or Mn were not evident in any samples. XRD iron lines identified giniite (iron phosphate hydrate) and quartz as the most probable substrate minerals of AS103. Analysis of AS103 Fe-S substrate samples showed akaganeite, Fe(III)O(OH), iron oxide hydroxide. Carbon disulfide extraction of the sulfur, followed by XRD analysis of the resulting iron residue, showed no minerals, i.e., no peaks, indicating that some of the iron oxide in the mixed Fe-S sample was amorphous.

Scans of yellow crystals sampled within a few centimeters directly above the reddish substrate of the AS103 sampling site revealed sulfur as the main peak (Fig. 3A) and Si as a smaller peak. In representative EDS scans, such as Fig. 3B, iron peaks were evident from Lα (0.705 keV), Lβ (0.718 keV), Kα (6.398 keV), and Kβ (7.057 keV). The third type of substrate sample had a reddish color deposited on yellow crystals and occurred in the midstream area of AS103. Crystals analyzed by EDS revealed a major S peak with minor peaks of several other elements, including Fe Lα (0.705 keV) and Lβ (0.718 keV) (Fig. 3C). A prominent sulfur peak was also obtained for AS101 and AS102. In contrast to AS103, however, Fe peaks did not appear in AS101 and AS102 samples. It is noteworthy that major sulfur peaks Kα (2.307 keV) and Kβ (2.464 keV) were always seen in spectra of yellow crystals, whether pure yellow (Fig. 3A) or red tinged (Fig. 3C), confirming the substrate color as mainly sulfur. EDS and XRD analyses of nearby RM were also carried out, and iron peaks were resolved in every EDS scan. Figure 3D is a representative example for RM that shows a major peak for silicon and minor iron and sulfur peaks in the EDS spectrum. Although a minor sulfur peak appeared (Fig. 3D), sulfur crystals were not seen by microscopic analysis and yellow color was not evident in the spring.

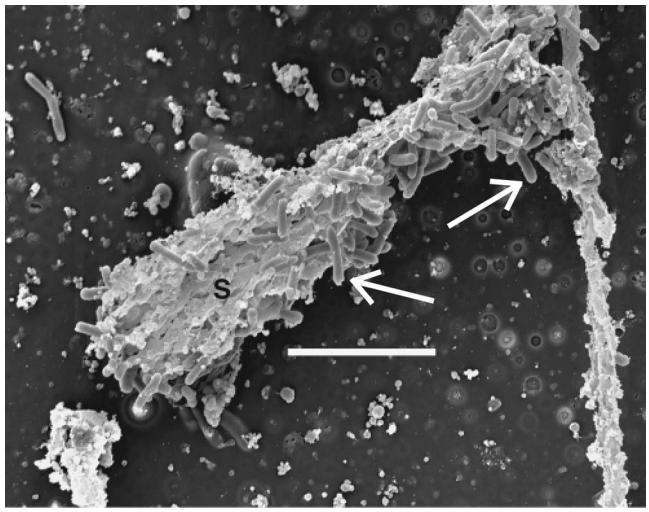

FIG. 2. Scanning electron micrograph of rod-shaped bacterial cells (arrows) attached to sulfur crystals (S) in a sulfur-dominated spring, AS102. Scale bar = 10 μm.

Because iron was identified in the substrate of AS103 and RM, we used a colorimetric assay for iron with 1,10-phenanthroline. With this assay, we examined RM samples and all samples from AS103, (i) yellow crystals, (ii) red-tinged yellow crystals, and (iii) reddish mineralized stream substrate, as well as the substrates from AS101 and AS102. Yellow crystals were present in AS101, AS102, and AS103; red-tinged yellow crystals were found in AS102 and AS103; while reddish sediments were characteristic of AS103 and RM. The results of the phenanthroline iron assay and the EDS analysis are summarized in Table 1.

RM substrates were positive for iron in the powdered substrates analyzed by 1,10-phenanthroline (Table 1). A reddish substrate deposit resembling iron was visibly evident over broad areas of the streambed and on smaller granular siliceous substrate particles. The reddish substrate and red-tinged crystals of AS103 were also positive for iron. At RM, XRD iden-

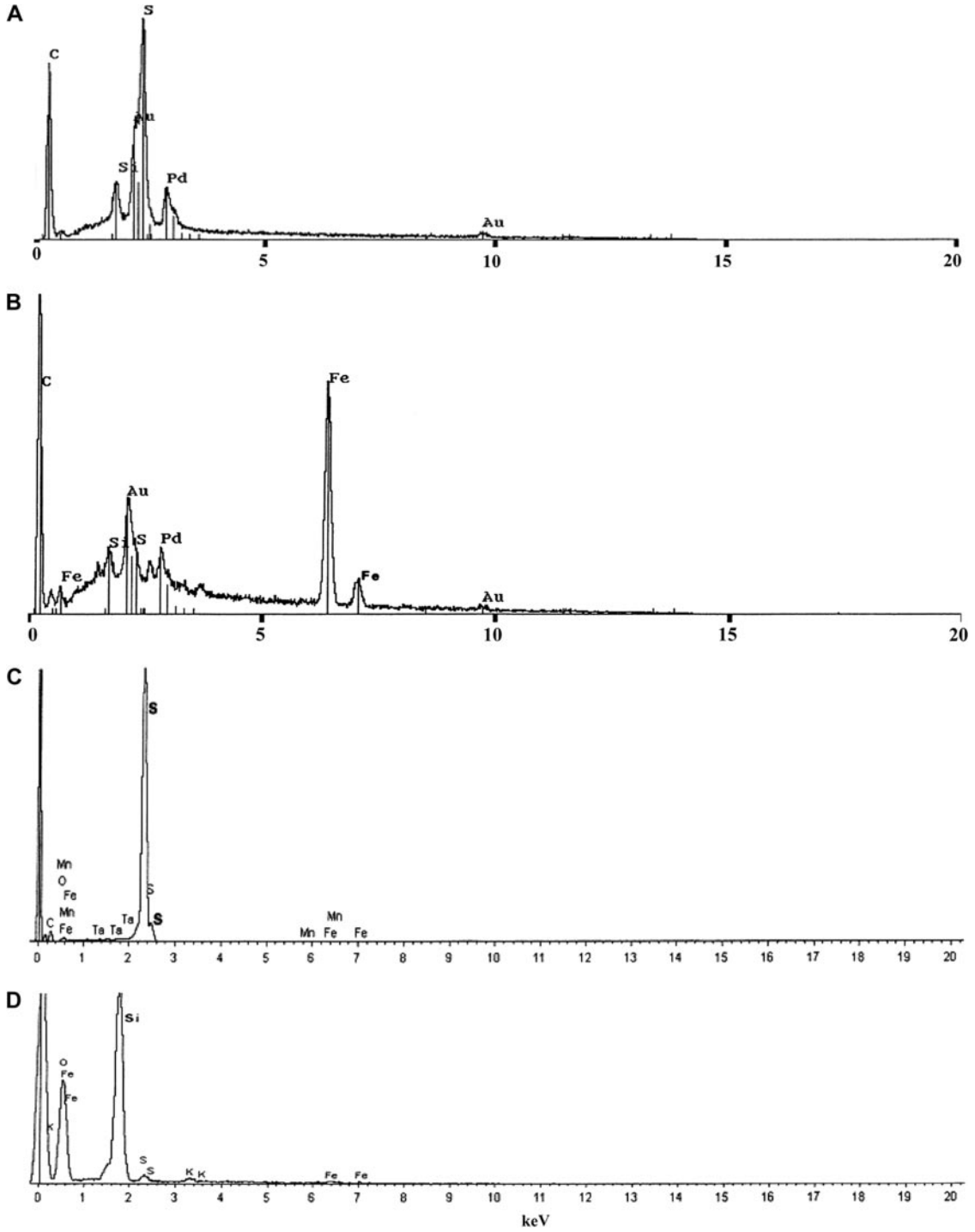

FIG. 3. Comparison of matched spectra (S and Fe) from AS103 and RM. Peaks from Au and Pd result from metal coating during SEM sample preparation. (A) Spectrum of yellow crystals sampled near the spring origin in a zone of turbulence in AS103. S peak is prominent; C and Si are also present. (B) Reddish-rust-hardened AS103 streambed sampled in the laminar flow of the spring just below the yellow crystals shown in panel A. In addition to Fe peaks, Si, S, and C also appear. Carbon-coated scans of the same sample revealed minor peaks of B, K, and Na and peaks of thallium (Tl) and Mn. (C) AS103 midstream reddish-tinged sulfur crystals. Prominent peaks for S with minor peaks for C, Fe, Mn, and Ta are shown. The sample was carbon coated. (D) RM sediment with red-rust deposits showing major Fe peaks. The sample was carbon coated.

tified giniite (iron phosphate hydrate) as the most probable mineral substrate in association with quartz.

Water chemistry. Table 2 presents the results of ICP analysis of the four springs for samples taken at the origin and analyzed for soluble elements, nutrients, and other properties. Samples were also taken from each of the four springs at each of the sampling temperatures and analyzed. These results (data not shown) were similar to the values shown in Table 2. Values for pH and E_h are strongly influenced by temperature and distance from the origin due to oxygenation, and these values varied in

TABLE 1. Substrate analytes in flowing springs in YNP

Sample site	Substrate[a]			X-ray detection[b]		
	S	Fe	Fe-S[c]	S	Fe	Fe-S[c]
AS101	+[d]	−	−	+	−	−
AS102	+	−	±	+	−	−
AS103	+	+	+	+	+	±
RM	−	+	−	±	+	−

[a] Nitric acid color change and 1,10-phenanthroline assay (9); sulfur was detected by color and SEM examination.
[b] XRD and EDS were used for analysis.
[c] Fe-S, iron deposited over sulfur.
[d] +, present as major or dominant chemical species in spring; ±, present in small but detectable quantities; −, absent.

each of the springs at the sampling sites. All of the springs sampled had iron; RM had the highest level of iron but had no H_2S or visible elemental sulfur. In contrast, the three AS sites were lower in iron and high in H_2S and elemental sulfur. All of the springs had iron that had changed only slightly in concentration from an earlier analysis performed in 1971 (6a) in conjunction with one of us (R.W.B.). Silicon was abundant in all four of the springs examined. Arsenic, although noted in nearby thermal springs at high concentrations (30),

TABLE 2. Analytes and other properties of water column samples from Yellowstone hot springs

Analyte	Concn[a] in samples from:			
	AS101	AS102	AS103	RM
Na	7.601	8.699	8.732	5.704
Ca	3.550	5.627	6.557	1.736
Al	10.73	16.39	16.07	24.58
Fe (total)	2.557	3.336	3.694	6.506
Si	303.0	307.1	298.1	382.6
B	0.0000	0.0000	0.0000	0.0000
K	21.22	23.41	26.95	17.12
Mg	1.797	2.568	2.577	0.3518
Zn	0.12686	0.06837	0.03169	0.03951
Mn	0.05056	0.09661	0.11346	0.06455
Mo	0.02871	0.02874	0.02841	0.02749
Se	0.0000	0.0000	0.0000	0.0000
Ni	0.0000	0.0000	0.0000	0.0000
Pb	0.0180	0.0144	0.0167	0.0192
Cr	0.0000	0.0000	0.0000	0.0000
Cd	0.0000	0.0000	0.0000	0.0000
Cu	0.0000	0.0000	0.0000	0.0000
Hg	0.0000	0.0000	0.0000	0.0000
As (total)	0.0141	0.0687	0.0541	0.1584
S	107.8	110.6	126.5	156.0
Sr	0.05569	0.06014	0.05795	0.04145
SO_4	265.0	260.0	265.0	250.0
NO_2/NO_3[b]	0.146	0.567	0.942	0.794
NH_4, N-NH_4	2.112	2.277	2.31	1.79
PO_4, P-PO_4	0.0076	0.008	0.003	0.0047
H_2S[c]	3.3	3.1	ND[d]	0.0
pH (origin)	1.08	1.14	1.22	1.19
E_h (mV, origin)	31.6	16.0	−10.0	21.0
E_h (mV, 75°C)		35	−4.3	247.2
E_h (mV, 70°C)	45.6	ND	ND	391.6
E_h (mV, 65°C)	296.2	ND	ND	397.5
E_h (mV, 60°C)	347.8	ND	ND	401.5
E_h (mV, Fe site)			−10	
E_h (mV, Fe/S site)			4	
Conductivity (μS/cm)	2,160	3,845	3,750	3,040
Temp (°C, origin)	75.8	77.0	76.1	93.5

[a] Values are reported as milligrams per liter unless indicated otherwise.
[b] N-NO_3 (micrograms per liter).
[c] Values are from reference 6a.
[d] ND, not determined.

TABLE 3. Distribution of Fe(II) and Fe(III) in the water columns and substrates of springs

Source of water and site	Concn (μM) of:	
	Fe(II)	Fe(III)
Water column		
AS101	15.604	41.494
AS102	0	78.353
AS103	29.502	33.065
RM	37.569	78.246
Substrate[a]		
AS101-70	0	50.353
AS102-70	0	20.680
AS103 Fe	116.1	903.9
AS103 Fe-S[b]	33.335	30.741
AS103S[c]	3.884	46.851
RM	21.404	452.686

[a] Values are based on 100-mg samples.
[b] Fe-S, iron deposited on sulfur.
[c] AS103 temperature was 75°C for all samples.

was present at conspicuously low levels (or absent) in both water column and sediment samples, as determined by ICP and substrate analyses (EDS and XRD) of all of the springs and sites sampled.

Sulfur was present as both H_2S and elemental sulfur at the three AS sites but was seen as a minor component at RM. Our EDS and chemical analyses (Table 1) confirm the presence of iron in the substrates at RM and AS103 and also on the midstream sulfur crystals of AS103. In contrast, the turbulent surface zone at the origin of AS103 contained sulfur crystals but without iron deposits. These results establish a basis for the chemical identities of the several springs and study sites sampled within AS103, between the several AS sites and between the study sites AS and RM. Table 3 presents the results of the distribution of iron species in the water column and sediment samples. The waters of AS were similar to each other in having both Fe(II) and Fe(III), with the exception of AS102, which had no Fe(II). AS103 had more Fe(II) than AS101. RM waters had the greatest amounts of both Fe(II) and Fe(III). The sulfur substrates of AS101, AS102, and AS103 had little or no Fe(II), with the exception of AS103 Fe-S, which had equal

TABLE 4. Major bacterial groups found in the springs relative to the basic chemistry of the sediment samples[a]

Spring	Temp (°C)	Chemistry[b]	Closest relative(s)
AS101	70	S	*Hydrogenobaculum* spp.
AS101	65	S	*Hydrogenobaculum* spp.
AS101	60	S	*Hydrogenobaculum* spp.
AS102	70	S	*Hydrogenobaculum* spp.
AS102	65	S	*Hydrogenobaculum* spp.
AS102	60	S	*Hydrogenobaculum* spp.
AS103	75	S	*Hydrogenobaculum* spp.
AS103	75	Fe-S	*Sulfobacillus disulfidooxidans*
AS103	75	Fe	Gram-positive acidophiles
RM	70	Fe	Gram-positive acidophiles
RM	65	Fe	Gram-positive acidophiles
RM	60	Fe	Gram-positive acidophiles

[a] Identifications were based on phylogenetic analysis.
[b] Fe denotes the presence of iron in that sample; S denotes the presence of sulfur. Fe-S denotes iron deposited on sulfur sediment.

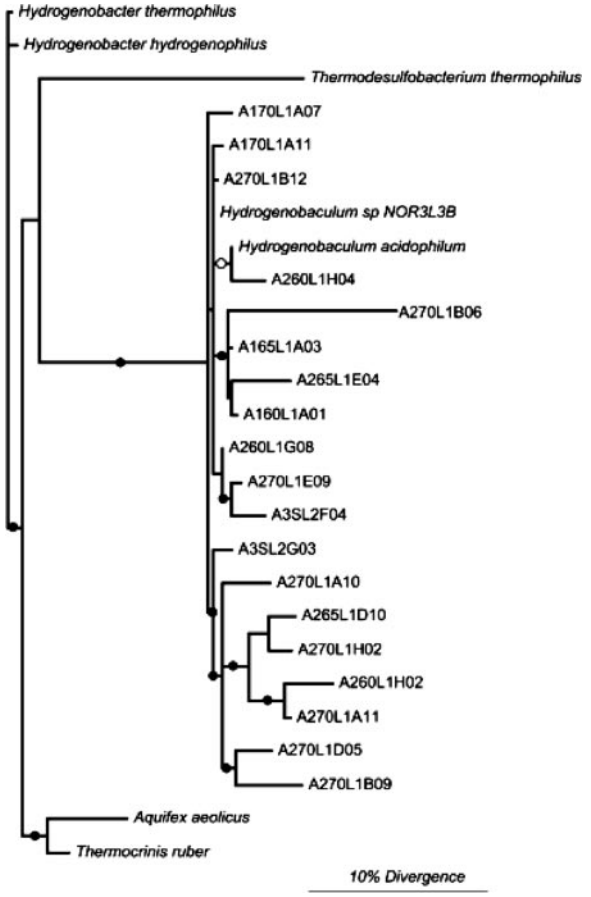

FIG. 4. Results of phylogenetic analyses with rRNA gene sequences determined from AS that were related to *Hydrogenobaculum* spp. The tree shown is the ML tree (ln = −2,118.2). MP and NJ analyses had similar topologies, and the minor differences among the trees found by each algorithm were not supported by bootstrap analyses. Filled circles indicate branches with ML and NJ bootstrap support of greater than 80%, and open circles indicate bootstrap support exceeding 50%. A1 = AS101; A2 = AS102; A3S = AS103 containing sulfur. The sampling temperatures were 70, 65, and 60°C for AS101 and AS102 and 75°C for AS103.

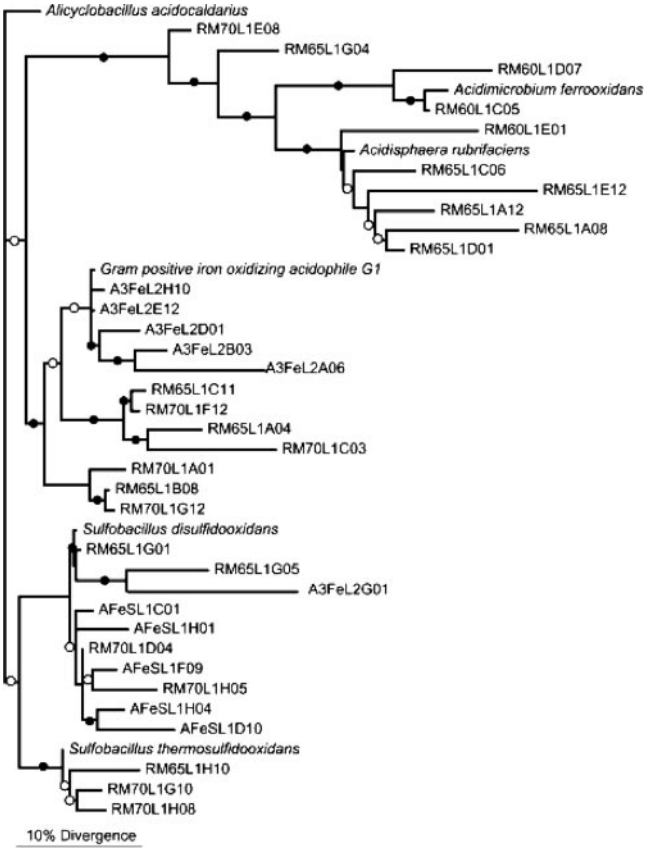

FIG. 5. Results of phylogenetic relationships of rRNA gene sequences determined from RM and AS103 Fe and AS103 Fe-S samples. The tree shown is the ML tree (ln = −2,882.6). MP and NJ analyses had similar topologies, and the minor differences among the trees found by each algorithm were not supported by bootstrap analyses. Filled circles indicate branches with ML and NJ bootstrap support of greater than 80%, and open circles indicate bootstrap support exceeding 50%. AFeS = AS103 containing iron and sulfur; A3Fe = AS103 containing iron.

amounts of Fe(II) and Fe(III) deposited on the sulfur substrate. All three sulfur substrates had moderate amounts of Fe(III). Both RM and AS103 Fe had significant amounts of Fe(II) and Fe(III) in the sediments.

PCR and sequencing. PCR amplifications of 16S rRNA genes were successful for all samples. *Hydrogenobaculum*-related species were the most prevalent kind of organisms in seven sulfur-rich samples (Table 4; Fig. 4). The DNA extraction procedure was monitored by DAPI and phase-contrast microscopy and was found to be complete (i.e., no intact cells were found). Analysis by distribution of sequences from clone libraries may be affected by PCR bias, and we used more PCR cycles than has been recommend in the literature (43). However, we found that 35 cycles was the minimum number of cycles needed to produce a band. The four samples with iron-rich deposits were dominated by rRNA gene sequences of uncultured bacteria related to gram-positive iron-oxidizing acidophiles (Table 4; Fig. 5). Iron-dominated deposits also contained rRNA gene sequences related to those of *Acidithiobacillus caldus*, *Acidisphaera rubrifaciens*, *Acidimicrobium ferrooxidans*, and *Sulfobacillus disulfidooxidans*. The mixed iron-sulfur sample from AS103 was dominated by uncultured bacteria related to *S. disulfidooxidans* (Table 4).

Statistical and phylogenetic analyses. Coverage for the different samples ranged from 43 to 96%. The values for the Shannon-Weiner index ranged from 0.83 to 3.67 (Table 5). Figures 4 and 5 report the results of phylogenetic analyses with representative rRNA gene sequences from the environments. Phylogenetic analysis found that rRNA gene sequences isolated from sulfur-dominated samples were from a diverse group of bacteria related to cultured *Hydrogenobaculum* spp. (ML, MP, and NJ bootstrap support, ≥80%; Fig. 4). Phylogenetic analysis found much greater diversity among sequences determined from iron-dominated sediments (RM, AS103 Fe) and mixed iron-sulfur sediments (Fig. 5). Again, there was strong bootstrap support for the relationships of these sequences to cultured organisms isolated from acidic springs (Fig. 5). The *P*-test comparisons found highly significant differences among most of the samples ($P \leq 0.0001$) (Table 6).

TABLE 5. Comparison of standard ecological and molecular estimates of sequence diversity for hydrothermal spring microbial communities[a]

Sample	No. of distinct sequences	No. of OTUs	Shannon-Weiner index	Coverage (%)
AS101-70	21	30	2.349	76
AS101-65	9	15	1.586	91
AS101-60	4	10	1.006	96
AS102-70	39	45	3.28	43
AS102-65	8	8	0.83	92
AS102-60	9	13	1.38	89
AS103 Fe	29	34	2.609	68
AS103 S	12	14	1.053	83
AS103 Fe-S[b]	24	42	3.274	69
RM-70	18	24	2.146	80
RM-65	48	56	3.672	44
RM-60	14	21	3.445	85

[a] All of the AS103 samples were collected at 75°C. The total number of clones screened was 96 clones per sample site, for a total of 1,152.
[b] Fe-S, iron deposited on sulfur sediment.

Φ_{ST} results also showed values close to 0 between the AS101 and AS102 springs, indicating significant genetic overlap, but the values were much higher in other comparisons, especially between AS and RM springs (Table 6). The Φ_{ST} values for comparisons of sediment samples within the mixed spring AS103 were close to 1, indicating that each sediment sample within AS103 harbored distinct microbial communities (Table 6).

PCA. The PCA of the Φ_{ST} data produced two factors, 1 and 2, that accounted for 85.7% and 7.8% of the variance, respectively, or 93.5% of the total (Fig. 6). The samples clustered strongly by chemistry, and springs with S separated from springs with Fe or Fe and S. Temperature had a secondary effect; within the same chemistry, 60 and 65°C samples clustered together, as did 70 and 75°C samples. Regression analysis of these two PCA factors on environmental variables confirmed these patterns (Table 7). Chemistry strongly correlated

TABLE 6. *P*-test results and Φ_{ST} values for community comparisons made in this study

Comparison	*P* value	Φ_{ST} value
Same temp, different chemistry, same spring		
AS103 Fe vs AS103 Fe-S	0.0001	0.433
AS103 S vs AS103 Fe-S	0.0001	0.794
Different temp, same chemistry, same spring		
RM-70 vs RM-65	0.0001	0.236
RM-70 vs RM-60	0.0001	0.524
RM-65 vs RM-60	0.0001	0.207
A102-70 vs AS102-65	NS[a]	0.090
A102-65 vs AS102-60	NS	0.009
A102-70 vs AS102-60	0.0008	0.086
A101-70 vs AS101-65	0.0001	0.075
Same temp, different chemistry, different springs		
RM-65 vs AS101-65	0.0001	0.593
RM-60 vs AS102-60	0.0001	0.681
Different temp, same chemistry, different springs		
RM-70 vs AS103 Fe	0.0001	0.274
RM-65 vs AS103 Fe	0.0001	0.244
A101-60 vs AS102-60	0.0001	0.124

[a] NS, not significant.

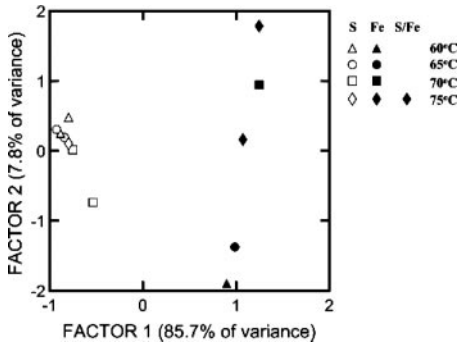

FIG. 6. Pairwise Φ_{ST} values between all samples were projected onto two dimensions by PCA, showing the genetic clustering of samples. Note that although the two factors are scaled equally, factor 2 only accounts for a minor part of the variance in Φ_{ST} values.

TABLE 7. General linear model regressions showing relationships between environmental variables and the two factors that resulted from the PCA analysis of Φ_{ST} values

Model	R^2	*P* value	Partial SSE[a]
Factor 1 = chemistry[b]	0.983	<0.0001	
Factor 1 = spring[c]	0.739	0.010	
Factor 1 = temp[d]	0.095	0.331	
Factor 1 =	0.983		
Iron[e]		<0.0001	3.85
Sulfur[e]		0.205	0.004
Factor 1 =	0.983		
Chemistry		<0.0001	2.69
Spring		0.984	0.004
Factor 1 =	0.995		
Chemistry		<0.0001	9.7
Temp		0.049	0.132
Factor 2 = chemistry	0.417	0.088	
Factor 2 = spring	0.320	0.352	
Factor 2 = temp	0.193	0.152	
Factor 2 =	0.417		
Iron		0.083	2.71
Sulfur		0.033	4.53

[a] Partial sums of squared errors (SSE) are shown for multiple regressions only.
[b] Chemistry was a categorical variable with three possible values (iron, sulfur, or both).
[c] Spring was a categorical variable with four possible values (RM, AS101, AS102, and AS103).
[d] Temperature was a continuous variable.
[e] Iron and sulfur were categorical variables with binary (yes or no) values.

with factor 1 and hence explains most of the genetic variability among the samples. Controlling for different chemistries, temperature was also significant, although it accounted for only a small part of the variability. While the hot springs significantly differed, this was not significant when chemistry was included in the model. When chemistry was decomposed into separate S and Fe variables, factor 1 was explained almost wholly by the presence or absence of Fe. Factor 2, on the other hand, was marginally related to chemistry, with S being the most important variable.

DISCUSSION

In order to understand the patterns of bacterial diversity of several Yellowstone spring sediments, it was critical to establish the chemical makeup of both the sediments and the water column. As expected, X-ray microanalysis (Table 1; Fig. 3A) and X-ray dot mapping (data not shown) found that the yellow sediments sampled in AS101, AS102, and AS103 were dominated by elemental sulfur (S^o). Despite the substantial differences in reduction potential between AS103 and AS101 or AS102 (Table 2), the S^o sediments were remarkably similar. The high concentration of S^o likely results from the abiotic conversion of dissolved H_2S to sulfur in the presence of oxygen, although we cannot rule out a minor contribution from biogenesis.

The red-rust sediments proved much more chemically diverse than the S^o sediments. As we predicted on the basis of color observations, the pure red-rust sediments of both RM and AS103 contained significant quantities of iron (Table 1; Fig. 3). Although the AS103 and RM sediments both contained iron (hence their color), they were very dissimilar in other respects. For instance, the AS103 red-rust sediments were iron phosphate hydrate mineralized as an aggregate of giniite and quartz, while RM sediments were more granular with iron phosphate hydrate deposited on silica granules (giniite and quartz).

Our water column analysis of AS103 showed that the fast flow rate of the spring maintains a low E_h within the channel that prevents S^o deposits from forming, except in turbulent surface zones near the origin or in the shallow, flat midstream area at the end of the channel. The RM source waters, on the other hand, do not contain H_2S and have approximately twice the quantity of iron as AS103. The E_h values were also very different between the springs, probably because of the substantial differences in temperature at the spring origins (93.5°C in RM versus 76.1°C in AS103). At approximately the same sampling temperature (~75°C), the E_h of RM was 247 mV (21 mV at the origin), while the E_h of AS103 was −4.3 mV (−10 mV at the origin).

Water column analysis showed that the source waters of the AS springs were chemically similar to one another in almost every respect, even in iron concentration (Table 2). This is not surprising, given their proximity, and suggests that the springs share subsurface source waters. The main difference between the AS101 and AS102 springs and the AS103 spring was in E_h (Table 2), which may explain the different patterns of mineral deposition between the AS101 and AS102 springs and AS103. The low E_h of AS103 favored iron deposition. The AS103 iron deposits contained Fe(II) in the form of giniite [iron phosphate, Fe(II), Fe(III), and phosphate] that would precipitate under the low-E_h condition observed near the origin of AS103 (Table 2). The higher E_h found at midstream in AS103 favored increased sulfur deposition because H_2S was converted into sulfur in the oxic zone.

Bacterial diversity and sediment chemistry. Our results strongly support the hypothesis (H1) that mineral chemistry, and thus metabolic potential, played a dominant role in shaping the composition of acidic thermal spring communities. In each of the spring sediments, the phylogenetic diversity correlated strongly with the predominant mineral chemistry of the sampled sediments despite some very clear differences in water column chemistry and reduction potentials (Table 2). Regardless of temperature and spring location, sulfur-rich sediments always supported a high proportion of rRNA gene sequences related to *Hydrogenobaculum* (Table 4). *Hydrogenobaculum* spp. grow chemolithoautotrophically by using sulfur, thiosulfate, or hydrogen as an electron donor (13, 20, 21, 40). Our phylogenetic analysis of rRNA gene clones uncovered a remarkable diversity of these organisms, including up to 19 uncultured new species (Fig. 4). This discovery suggests that relatives of *Hydrogenobaculum* may be more widespread and diverse than previously suspected. *Hydrogenobaculum* spp. also have a broad growth range spanning temperatures from 55 to 80°C (13), which corresponds well with the temperatures of our spring sediments and the group as a whole, which can tolerate a wide range of physiochemical diversity in their environments (42).

Hydrogenobaculum spp. have also been found abundantly in other high-temperature acid-sulfate springs in YNP (11, 25).

In contrast to the sulfur sediments, the iron-rich sediments contained a substantial diversity of sequences (~23 potential new bacterial species) from uncultured bacteria related to iron-oxidizing organisms (Fig. 5). These new sequences included relatives of a cultured gram-positive iron oxidizer from YNP and sequences related to *A. caldus*, *A. ferrooxidans*, and *A. rubrifaciens*, as well as several *Sulfobacillus* species known to oxidize iron, among other compounds (5, 34, 35). A recent study of hydrocarbon seeps at YNP's Rainbow Springs found relatives of *Acidisphaera* spp. and *Acidithiobacillus* spp. in these extremely acidic, Fe-rich soils (18). All of these cultured relatives grow well at low pH and moderately high temperatures and have been found in other geothermal habitats. The RM sediments contained organisms closely related to those found in the iron-rich AS103 sediments (Fig. 5). This pattern held in spite of strong differences in water column chemistry and E_h between AS103 and RM and a geographic distance of ~2 km.

Indeed, the most revealing comparisons made in this study with regard to mineral chemistry were among the sediment samples collected within the same spring, AS103. The three AS103 samples we collected from iron-rich, sulfur-rich, and mixed iron-sulfur sediments were dissimilar in terms of organismal diversity yet were quite similar to other spring sediments with similar mineral chemistry (Table 4). The rRNA gene sequences from the iron-rich AS103 sediments were closely related to sequences found in the iron-rich RM samples (Table 6; Fig. 5). Similarly, the rRNA gene sequences from the sulfur-rich AS103 sample were closely related to sequences in AS101 and AS102 (Table 6; Fig. 4). Interestingly, the sequences isolated from the mixed AS103 Fe-S sediments clustered with RM

sequences collected at 70°C and were related to *S. disulfidooxidans* (Fig. 5). *S. disulfidooxidans* is an acidophilic, gram-positive, aerobic microorganism with an optimal growth pH of around 1.5 to 2.5 that utilizes elemental iron and sulfur as energy sources (26) in most, but not all, habitats (12).

The novel application of PCA to Φ_{ST} data proved fruitful for testing the hypothesis that mineral chemistry, per se, plays the dominant role in structuring these sediment communities. Most of the genetic variability between samples was described by a single factor (Fig. 6). Factor 1 accounted for 85.7% of the Φ_{ST} variance, and chemistry, in turn, accounted for 98.3% of the variance in factor 1 (Fig. 6; Table 7). Clearly, sediment chemistry (H1) was the primary factor in structuring these microbial communities. However, according to the multiple regression of factor 1 on chemistry and temperature, temperature (H2) did have a significant, albeit secondary, effect on diversity (Table 7).

P-test and Φ_{ST} results, especially for comparisons of bacterial diversity in AS103 sediments, strongly supported the hypothesis that the mineral chemistry of sediment deposits plays the predominant role in determining bacterial community diversity. Within-spring comparisons of AS103 Fe or AS103 S with the mixed AS103 Fe-S spring also showed significant differences in bacterial diversity ($P < 0.0001$ by *P* test; Table 6), even though the water column chemistry, temperature, and pH were identical. This result strongly suggests that each predominating chemical environment selects for distinct phylogenetic lineages of bacteria.

As with all studies of microbial environments that use PCR amplification with universal primers, we could not rule out the possibility that "primer bias" affected the diversity of the sequences found in our clone libraries (46). Some bacterial sequences may have been proportionally overrepresented in our clone library and some underrepresented (or missed altogether), and such a bias may have affected our diversity measurements. However, our diversity abundance patterns appear to be consistent with other culture-independent studies of similar geothermal habitats that used different sets of universal primers (30, 42).

Substrate metabolism and diversity. The analysis of Fe(II) and Fe(III) in the substrates indicated that iron was available as both an electron donor and acceptor under oxygen-limited or anaerobic conditions. rRNA gene sequences related to several iron-oxidizing or -reducing species, e.g., *A. ferrooxidans*, *Sulfobacillus thermosulfidooxidans*, *A. rubrifacien*s, and *A. caldus*, were found in the clone libraries from RM and AS103 Fe. Cultured isolates of these bacterial species can reduce Fe(III) in ferric-iron-containing substrates in microaerophilic or anaerobic substrates such as those found in the two iron springs (Table 3). This metabolic potential would give populations attached to these sediments ready access to a terminal electron acceptor under oxygen-limited conditions. Johnson et al. (26) showed that cultured isolates of *A. rubrifaciens* can use Fe(III)-containing substrates as electron acceptors. *A. ferrooxidans* and *S. thermosulfidooxidans* can oxidize or reduce iron, and *A. caldus* can oxidize Fe(II) or sulfur (26). The ratios of Fe(II)/Fe(III) in the sediments of RM and AS103 Fe suggest that these substrates would support either oxidation of Fe(II) or reduction of Fe(III) under oxygen-limited conditions (5).

The ratio of Fe(II)/Fe(III) serves as a proxy for the E_h values of the two AS103 sulfur substrates. The Fe(II)/Fe(III) ratio of S° gave a low ratio of 0.083 (i.e., a high E_h value) for sulfur at the AS103 origin, where only *Hydrogenobaculum*-related sequences were identified. The AS103 midstream mixed iron-and-sulfur sediments (Fe-S) had a ratio of 1.084 (i.e., a low E_h value), and these sediments contained sequences related to *S. disulfidooxidans*. *S. disulfidooxidans* can oxidize pyrite, disulfide, S°, and iron (5, 26). H_2S oxidation by *S. disulfidooxidans* could occur with Fe(III) reduction in the sediments, or it could be coupled to O_2 as an electron acceptor in a more energetically favorable reaction.

The 16S sequences from the sulfur-dominated sediments in AS101, AS102, and AS103 were related to *Aquificales* isolated from low-temperature (60 to 80°C) and pH ~3 habitats, which are represented by *Hydrogenobaculum* (39, 42). These organisms physically attach to sulfur, and this is a possible energy source supporting this group with O_2 as the terminal electron acceptor. However, H_2 oxidation cannot be ruled out because organisms in the clone library were closely related to *Hydrogenobaculum* sp. strain NOR3L3B (13), which is a chemolithotroph that appears to use H_2 as an energy source. Under microaerophilic conditions, this organism also requires sulfur for growth. Thus, it is not surprising that we found bacteria attached to sulfur crystals when sulfur sediment samples from AS101 and AS102 were examined with a universal bacterial fluorescein isothiocyanate probe by fluorescence in situ hybridization (data not shown). Similar populations related to *Hydrogenobaculum* sp. strain NOR3L3B have been identified in clone libraries from sulfur substrates in nearby Norris Basin (30).

Bacterial diversity and temperature. *P* tests found significant differences in the phylogenetic composition of RM communities from sediments that differed by 5°C (Table 6). Temperature differences (H2) appear to select for different phylogenetic groups in the RM spring, although we did not find distinct monophyletic groups at each temperature (Fig. 5). *P* tests also found significant differences between AS spring sediment samples at 70°C compared with 65°C or 60°C samples from the same springs (Table 6). Φ_{ST} values tended to be smaller between 65°C and 60°C sediment samples than between 60°C and 70°C or 65°C and 70°C samples (Table 6). A closer look at the phylogenetic relationships with AS101 and AS102 revealed substantial phylogenetic overlap among the rRNA gene sequences determined at 65°C and 60°C, but the 70°C sample sequences mostly belong to a separate group that may be more thermally tolerant (Fig. 4).

Interestingly, we found that microbial diversity was greater in the 70°C AS101 and AS102 sediments than in the 60°C or 65°C sediments (Table 6). These findings concur with earlier studies of the same springs in 1973 and 2001. Weiss (50) used C^{14}-labeled glutamate to count cell concentrations in AS101 and AS102 and found that cell counts peaked at around 70°C and dropped off at both higher and lower temperatures. Cell counts of AS102 sediments showed the same pattern again in 2001 (data not shown). In other springs, microbial diversity typically decreases as the temperature increases (41). The fact that we did not see a drop-off in the diversity in the highest-temperature sediments we sampled (70°C) was probably because this temperature was not particularly high compared to those in other studies (85°C and higher) (41). Incidentally, the

temperature profile of the AS springs had declined significantly, from a high of close to 90°C in 1973 to 75 to 79°C by time of this study, making it impossible to sample diversity at equivalently high temperatures at AS (75°C) and RM (93.5°C). Such a temperature change could possibly explain the lower diversity at 65 and 60°C.

Overall, we were encouraged to discover that our microbial diversity estimates correspond so well to the cell count data from previous studies. Greater cell abundance may allow for increased diversity increases because of cell death, interactions, and cross-feeding. The high bacterial diversity and abundance found at 70°C suggest that this temperature may represent a "zone of overlap" between moderate and more extreme thermophiles. A closer look at the diversity measurements supported the idea that there is greater overlap (i.e., lower Φ_{ST} values) between the 65°C and 70°C sediments compared with 70°C to 60°C or 65°C to 60°C in AS101, AS102, and RM (Table 6). Phylogenetic analysis indicated that the 70°C sediments contained different lineages of bacteria not found in the lower-temperature sediments (Fig. 1). One possibility is that these lineages were present in springs when the temperatures were higher and represent derivatives of strains from earlier times.

Although this study focused exclusively on bacterial diversity, archaea are also present in these springs and may have a significant influence on bacterial diversity (50; unpublished data). For example, archaeal growth and metabolic products could be utilized by bacteria for cross-feeding and metabolism at many levels and could influence bacterial diversity and vice versa. This will be an important area of investigation for future research in these springs.

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ADDENDUM IN PROOF

Sulfobacillus disulfidooxidans is now classified in the genus *Alicyclobacillus* (G. I. Karavaiko, T. I. Bogdanova, T. P. Tourova, T. F. Kondrat'eva, I. A. Tsaplina, M. A. Egorova, E. N. Krasil'nikova, and L. M. Zakharchuk, Int. J. Syst. Evol. Microbiol. **55:**941–947, 2005).

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