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Molecular Characterization of the Diversity and Distribution of a Thermal Spring Microbial Community by Using rRNA and Metabolic Genes▽†

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The diversity and distribution of a bacterial community from Coffee Pots Hot Spring, a thermal spring in Yellowstone National Park with a temperature range of 39.3 to 74.1°C and pH range of 5.75 to 6.91, were investigated by sequencing cloned PCR products and quantitative PCR (qPCR) of 16S rRNA and metabolic genes. The spring was inhabited by three *Aquificae* genera—*Thermocrinis*, *Hydrogenobaculum*, and *Sulfurihydrogenibium*—and members of the *Alpha-*, *Beta-*, and *Gammaproteobacteria*, *Firmicutes*, *Acidobacteria*, *Deinococcus-Thermus*, and candidate division OP5. The in situ chemical affinities were calculated for 41 potential metabolic reactions using measured environmental parameters and a range of hydrogen and oxygen concentrations. Reactions that use oxygen, ferric iron, sulfur, and nitrate as electron acceptors were predicted to be the most energetically favorable, while reactions using sulfate were expected to be less favorable. Samples were screened for genes used in ammonia oxidation (*amoA*, bacterial gene only), the reductive tricarboxylic acid (rTCA) cycle (*aclB*), the Calvin cycle (*cbbM*), sulfate reduction (*dsrAB*), nitrogen fixation (*nifH*), nitrite reduction (*nirK*), and sulfide oxidation (*soxEF1*) by PCR. Genes for carbon fixation by the rTCA cycle and nitrogen fixation were detected. All *aclB* sequences were phylogenetically related and spatially correlated to *Sulfurihydrogenibium* 16S rRNA gene sequences using qPCR ($R^2 = 0.99$). This result supports the recent finding of citrate cleavage by enzymes other than ATP citrate lyase in the rTCA cycle of the *Aquificaceae* family. We briefly consider potential biochemical mechanisms that may allow *Sulfurihydrogenibium* and *Thermocrinis* to codominate some hydrothermal environments.

The *Aquificales* are a strictly thermophilic bacterial lineage that has been recovered from hydrothermal systems worldwide (9, 19, 26, 42, 52, 53, 59). This lineage has attracted much interest in the last 20 years because of its proposed deep phylogenetic position (10, 16, 29, 49). Culturing of the *Aquificales* has demonstrated that they are largely chemolithoautotrophs that utilize H_2 to reduce O_2 (Knallgas reaction) (30). However, culturing data are often inadequate to understand natural communities as the availability of metabolic substrates and energy yield depend on factors such as geochemistry and microbial community composition, which vary within and between hydrothermal environments.

Although *Aquificales* are chemolithoautotrophs, culture studies and environmental sampling suggest that they use a diversity of metabolic reactions. Hydrogen oxidation is one of the most exergonic reactions in *Aquificales*-dominated hot springs in Yellowstone National Park (42, 59), but most species are able to oxidize elemental sulfur, thiosulfate, or ferrous iron and reduce nitrate, ferric iron, arsenate, selenate, selenite, or elemental sulfur in addition to or instead of the Knallgas reaction to yield energy (1, 2, 19, 24, 27, 28, 36, 37, 44–46, 64–66, 69). Furthermore, there is a pattern between the general metabolic strategy and habitat of each species. Species isolated from terrestrial hot springs and compost are the only *Aquificales* capable of using organic compounds as carbon and energy sources. For example, *Thermocrinis ruber* and *Sulfurihydrogenibium* species, which are often the dominant species in high-temperature, near-neutral hot springs (9, 26, 42, 58), are facultative heterotrophs (28, 45). In contrast, all marine *Aquificales*, including *Hydrogenothermus marinus*, all *Aquifex* species, all *Persephonella* species, and all species in the *Desulfurobacteriaceae* family (incertae sedis), are obligate autotrophs (2, 24, 29, 36, 46, 61, 66, 69). Only two species fall outside these categories: the chemolithoautotroph *Hydrogenivirga caldilitoris* isolated from a coastal hot spring, and the facultative chemolithoautotroph *Hydrogenobacter subterraneus*, isolated from the deep subsurface (22, 44, 65).

Environmental surveys of the *Aquificales* suggest that their metabolic capabilities play important roles in biogeochemical cycles. Culture studies indicate that *Aquificales* can oxidize sulfur to sulfuric acid or reduce it to hydrogen sulfide, and molecular analyses indicate that *Aquificae* are a dominant phylum of high-sulfide hot springs (28, 29, 58). Genes for thiosulfate oxidation have been identified in the *Aquifex aeolicus*

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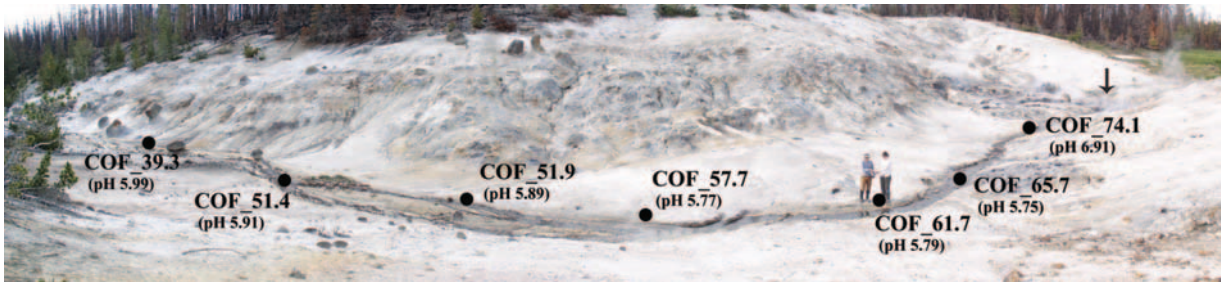

FIG. 1. Panoramic view of Coffee Pots Hot Spring (Mirror Plateau, northeastern quadrant of Yellowstone National Park). Image is a compilation of overlapping individual digital photographs with brightness uniformly increased in Adobe Photoshop 10. Sample sites are indicated by triangles with pH in parentheses. The arrow indicates the source of the spring.

genome (16) and amplified from a *Sulfurihydrogenibium* species (GenBank accession number AB254380). Nitrate reduction is present in at least one species each in the *Aquifex*, *Hydrogenobacter*, *Persephonella*, *Hydrogenivirga*, and *Sulfurihydrogenibium* genera, while nitrite reduction has been demonstrated in *Hydrogenobacter thermophilus* TK-6 through the characterization of the *nirS* gene and by weak growth of *Aquifex pyrophilus* with nitrite as the only electron acceptor (24, 29, 35, 44, 46, 62, 64). Additionally, *Aquificales* have been postulated to be primary producers in environments where photosynthesis is temperature limited. The reductive tricarboxylic acid (rTCA) cycle has recently been identified as the carbon fixation mechanism used by chemolithoautotrophs in all three *Aquificales* families (32).

We investigated the diversity and distribution of thermophilic bacteria and the potential metabolic processes of Coffee Pots Hot Spring, a remote spring located on Yellowstone's Mirror Plateau. We detected *Sulfurihydrogenibium* and *Thermocrinis* in a single sample using 16S rRNA gene libraries and determined that both species were abundant throughout Coffee Pots using quantitative PCR (qPCR) assays specific for these genera. We investigated the spatial patterns of these two species in relation to genes used in ammonia oxidation (*amoA*, bacterial gene only), the rTCA cycle (*aclB*), the Calvin cycle (*cbbM*), sulfate reduction (*dsrAB*), nitrogen fixation (*nifH*), nitrite reduction (*nirK*), and sulfide oxidation (*soxEF1*) using PCR and qPCR (12, 13, 20, 41, 55, 68, 70). Overall, we found a diversity of sequences indicative of carbon fixation by the rTCA cycle and linked their distribution to the abundance of *Sulfurihydrogenibium* 16S rRNA gene sequences.

MATERIALS AND METHODS

Sample site and collection. Samples were collected in July 2003 from Coffee Pots Hot Spring (UTM Easting: 554771.2; UTM Northing: 4955983.7) at seven points along a thermal gradient with a temperature range of 39.3 to 74.1°C and pH range of 5.75 to 6.91 (Fig. 1). Sample names represent the temperature at which they were collected. Coffee Pots is within the northwestern quadrant of Yellowstone National Park on the northwestern edge of the Mirror Plateau and consists of two main areas located in a north-south orientation. We sampled the lower area, a long stream that flows east to west. Filamentous biomass was collected in triplicate for all seven points using sterile syringes. Two replicates (R1 and R2) were stored in an equal volume of sucrose lysis buffer (SLB; 20 mM EDTA, 200 mM NaCl, 0.75 M sucrose, 50 mM Tris-HCl, pH 9.0), and the third was preserved in an equal volume of guanidine isothiocyanate medium (5 M guanidine isothiocyanate, 50 mM Tris, pH 7.4, 25 mM EDTA, pH 8, 0.8% 2-mercaptoethanol). Replicates at all points were taken within 2 cm of each other. Because of the remote location, samples were held at ambient air temperature (~10 to 26°C) for up to 10 days before they were stored at −80°C. Independent experiments have indicated that storage of samples in SLB at ambient air temperatures does not result in a loss of DNA or diversity in the samples relative to samples immediately frozen in liquid nitrogen (K. Mitchell and C. Takacs-Vesbach, unpublished data). However, we cannot rule out any long-term storage effect on gene abundances. Presumably, any decreases would be uniform across all members of the community.

Geochemistry measurements and metabolic energetic calculations. Temperature and pH were measured using a Thermo Orion 290A+ meter, and conductivity was measured using a WTW 340i meter. Spring water was collected for geochemical analysis at sample COF_65.7 but not at the other sites along the stream. The water directly overlying the biomass was sampled by syringe and filtered (0.2-μm pore size) into appropriately washed polyethylene or glass bottles. Water samples were preserved when necessary as appropriate for the analysis to be performed. Briefly, water for cation determination was preserved in 1% concentrated redisitilled HNO_3, the iron and arsenic species and dissolved organic carbon samples were stored in 1% 6N HCl, and the ammonium sample was preserved in 0.1% H_2SO_4. Geochemical analyses, including anions, cations, trace metals, and nutrients were conducted using standard U.S. Geological Survey (USGS) methods (39). Sulfide concentration was measured spectrophotometrically in the field on triplicate water samples using a Hach kit. Sample was diluted with deionized water when necessary, and a temperature correction was applied to the measurement following the method of McCleskey et al. (39). Anions and cations were determined using ion chromatography and inductively coupled plasma optical emission spectrometry; alkalinity was measured by titration, total and ferrous iron was determined colorimetrically by the ferrozine method, arsenic species were measured by atomic absorption spectroscopy, and dissolved organic carbon was determined by the wet oxidation method using a total organic carbon analyzer. Details of the analyses are given in McCleskey et al. (39).

Activities and speciation of metabolically important chemical compounds were calculated with both PHREEQC Interactive (version 2; USGS, www.brr.cr.usgs.gov/projects/GWC_coupled/phreeqc/) and Geochemist's Workbench (version 7.0; Rockware), which yielded virtually identical results. The activities were calculated for the redox reactions by decoupling the reactions to better approximate the disequilibrium found in natural environments. These activities were used in conjunction with thermodynamic data for high temperatures (3) to calculate the chemical affinity as previously described (57). Our calculation method was compared with previous results from Obsidian Pool: for all 41 reactions considered here, the chemical affinities were well within the ranges previously reported (see supplemental material) (3, 57). The chemical affinity is a measure of the disequilibrium state of many oxidation-reduction reactions that potentially serve as energy pathways for microbial metabolism under the specific sample location conditions. As gas chemistry was not available for Coffee Pots, we ran a number of models with various combinations of H_2 and O_2 (see Fig. 2 for accompanying model conditions and reaction list). Models 1 to 4 examine low (4.1×10^{-6} ppm), medium (2.05×10^{-5} ppm), medium-high (2.05×10^{-4} ppm), and high (6.67×10^{-4} ppm) concentrations of hydrogen encompassing the ranges reported for Yellowstone hot springs (59) for an oxygen concentration of 0.1 ppm. We evaluate the effect of varying O_2 in models 5, 3, and 6 (0.01, 0.1, and 0.5 ppm, respectively) at a fixed H_2 of 2.05×10^{-4} ppm. Because nitrate concentrations were below the analytical detection limit, we used the detection limit value (0.1 ppm) in all model runs.

DNA extraction. Nucleic acids were extracted using a variation of the cetyltrimethylammonium bromide (CTAB) method (73). CTAB buffer (400 μl of 1% CTAB, 0.75 M NaCl, 50 mM Tris, pH 8, 10 mM EDTA) and proteinase K (final concentration, 100 μg/ml) were added to 200 μl of SLB-preserved sample and

TABLE 1. Primers used for the detection of metabolic genes in this study

Pathway	Gene	Primer (5′–3′)	Size (bp)	Reference	Amplification conditions
Ammonia oxidation (bacterial gene only)	*amoA*	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	491	55	94°C 5 min; 42 cycles of 94°C for 60 s, 55°C for 90 s, 72°C for 90 s; 60°C for 90 s, 72°C for 10 min
rTCA cycle	*aclB*	TGGACMATGGTDGCYGGKGGT ATAGTTKGGSCCACCTCTTC	333	12	35 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 60 s
Calvin cycle (RuBisCO form II)	*cbbM*	ATCATCAARCCSAARCTSGGCCTGC GTCCC MGAGGTGACSGCRCCGTGRCCRG CMCGRTG	400	20	94°C for 2 min; 30 cycles of 94°C for 60 s, 62°C for 60 s, 72°C for 60 s; 72°C for 15 min
Sulfate reduction (dissimilatory)	*dsrAB*	ACSCACTGGAAGCACG GTGTAGCAGTTACCGCA	1900	70	94°C for 15 s; 30 cycles of 94°C for 15 s, 53°C for 20 s, 72°C for 54 s; 72°C for 60 s
Nitrogen fixation	*nifH*	GGHAARGGHGGHATHGGNAARTC GGCATNGCRAANCCVCCRCANAC	400	41	94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s; 72°C for 10 min
Nitrite reduction (dissimilatory)	*nirK*	CGTCTAYCAYTCCGCVCC GCCTCGATCAGRTTRTGG	540	13	95°C for 2 min, 30 cycles of 94°C for 30 s, 43°C for 40 s, 72°C for 40 s; 72°C for 7 min
Sulfide oxidation (thiosulfate utilization gene cluster)	*soxEF1*	TGYGCIGGITGYCAYGGIAC GCIGCRTGCCAYTCRATCA	993	68	94°C for 5 min; 30 cycles of 94°C for 30 s, 51°C for 30 s, 51°C for 30 s, 72°C for 60 s; 72°C for 7 min

incubated for 1.25 h at 60°C. Sodium dodecyl sulfate was then added to a final concentration of 2%, and samples were incubated for 1 h. DNA was extracted once with an equal amount of phenol-chloroform-isoamyl alcohol (50:49:1) and then extracted twice with an equal volume of chloroform. DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of 95% ethanol, followed by incubation at −20°C for 1 to 24 h. The samples were then centrifuged for 45 min (~21,000 × *g*), washed in 70% ethanol, and resuspended in 10 mM filter-sterilized Tris buffer, pH 8.0.

Gene amplification and sequencing. The 16S rRNA gene was amplified from one extraction of each SLB replicate of sample COF_65.7 by 50 μl of PCR mixture containing 5 μl of 10× buffer (Promega buffer B with 1.5 mM $MgCl_2$), a 12.5 mM concentration of each deoxynucleoside triphosphate (BioLine USA, Inc.), 20 pmol each of the 8F and 1492R primers, 2.5 U of *Taq* polymerase (Promega), and approximately 50 ng of DNA. The PCR was incubated for 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 50°C, and 90 s at 72°C, with a final extension of 72°C for 7 min. PCR was used to detect *amoA*, *aclB*, *cbbM*, *dsrAB*, *nifH*, *nirK*, and *soxEF1* genes using the primers and amplification conditions listed in Table 1. Temperature gradient and touchdown thermocycling programs were used to optimize the annealing temperature for each primer set. Appropriate positive controls were identified for each primer set and used in every PCR. The 50-μl PCR mixtures contained 5 μl of 10× buffer, a 12.5 mM concentration of each deoxynucleoside triphosphate, 3 μl of 2% bovine serum albumin, 2.5 U of DNA *Taq* polymerase, and 10 to 50 ng of DNA with the following amounts of primers: 40 pmol for *amoA*, 80 pmol for *aclB*, 100 pmol for *cbbM*, 40 pmol for *dsrAB*, 140 pmol for *nifH*, 80 pmol for *nirK*, and 100 pmol for *soxEF1*.

PCR products were spin purified using a DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA) and cloned using a TOPO-TA cloning kit (Invitrogen Corp., Carlsbad, CA). Clones were grown on kanamycin-selective plates, and plasmids from 92 to 103 colonies (per PCR) were harvested and isolated using an Eppendorf Perfectprep Plasmid 96 Vac Direct kit. Restriction fragment length polymorphism analysis was performed for each gene by reamplifying the inserted gene using vector-specific primers (M13F and M13R) in a 50-μl PCR mixture. The amplified genes were digested with restriction enzymes (New England Biolabs) for 6 h in 25-μl reaction mixtures containing 2.5 μl of 10× NEB-2 buffer, 2.5 μl of 1% Triton X-100, and 12 μl of PCR product with one unit of HinP1I and 0.5 units of MspI for the 16S rRNA gene, one unit each of DdeI and MseI and 0.25 μl of 100 μg/ml bovine serum albumin for the *aclB* gene, and 10 units each of MspI, EcoRI, and HindIII for the *nifH* gene. The digested genes were separated on agarose gels consisting of 3% agarose in 1× Tris-acetate-EDTA buffer for ~2 h at 2.5 V cm^{-1} of electrode length. Representative clones of each unique banding pattern were fully sequenced in both directions using primers M13F and M13R and internal primers using a BigDye terminator cycle sequencing kit (PE Applied Biosystems). The COF_65.7 R2 and six *aclB* libraries were screened for unique clones by sequencing the entire library with primer 8F (for the 16S rRNA gene) or M13F (for the *aclB* gene). Clones that were at least 2% dissimilar from other clones in the library were fully sequenced and included in phylogenetic analysis.

qPCR. qPCR assays were designed to quantify the number of gene copies of *aclB* and 16S rRNA gene sequences specific for *Thermocrinis*, *Sulfurihydrogenibium*, and a divergent sequence ("Toll" clone) detected in the R1 COF_65.7 clone library. New primers and fluorogenic TaqMan probes with minor groove binders on the 3′ end (Applied Biosystems) were designed for each assay (Table 2). The *aclB*, *Thermocrinis*, and Toll primers and probes were designed from an alignment of sequences amplified from Coffee Pots sample BLAST searches, and alignment with other *aclB* sequences using NCBI's bl2seq tool indicated that this primer and probe matched other *Sulfurihydrogenibium* sequences from Yellowstone but not other *Aquificales* species. The ThermoR primer matches mostly *Thermocrinis* sequences from Yellowstone springs but also matched sequences from a spring in the Alvord Desert Basin of Oregon (GenBank accession number DQ645256) and a hot spring from Nevada (GenBank accession number DQ490016.1). *Sulfurihydrogenibium*-specific primers appropriate for qPCR under our conditions could not be designed, so bacterial-specific primers were used in conjunction with a *Sulfurihydrogenibium*-specific probe already described (52). Although the primers could amplify any bacterial or archaeal 16S rRNA gene, fluorescence would be detected only from sequences to which the *Sulfurihydrogenibium*-specific probe had also bound.

Primers and probes were used in 25-μl qPCR mixtures containing 12.5 μl of TaqMan Universal PCR Master Mix without AmpErase uracyl *N*-glycosylase, 10 pmol each of the forward and reverse primers, 0.625 pmol of TaqMan probe, 0.5 U of *Taq* DNA polymerase, and 10 to 50 ng of DNA. Each reaction mixture was spiked with Promega *Taq* to increase the assay's resistance to inhibitors, a common problem in environmental samples. The 16S rRNA gene assays were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s, and the *aclB* assay was incubated for 35 cycles of 94°C for 30 s, 51°C for 30 s, and 72°C for 1 min using an ABI Prism Sequence Detection System. To ensure specificity of the primers and probes, the *Sulfurihydrogenibium* assay was tested on *Thermocrinis*-containing environmental samples, and the *Thermocrinis* assay was tested on *Sulfurihydrogenibium*-containing environmental samples; both results were negative. Three replicate reactions were performed and averaged for each assay.

Standards for the *Thermocrinis*, Toll, and *aclB* assays were generated by amplifying environmental DNA in 50-μl PCR mixtures with assay-specific primers, spin-purifying the PCR products, and quantifying DNA with an ND-1000 Spectrophotometer (Nanodrop Technologies). For the *Sulfurihydrogenibium* 16S rRNA gene, bacteria-specific primers 515F (5′-GTGCCAGCMGCCGCGGTA

TABLE 2. Primers and probes used for qPCR detection in this study

Gene	Primer				Probe	
	Name	Sequence (5′–3′)	Size (bp)	Source, reference, or type[b]	Sequence	Reference or source
Thermocrinis 16S rRNA	8F		174	B	TCCCAAGGGCAGGTTACTCA	This study
	ThermoR	GGCCACCTTTCACCGGGAGA		This study		
Sulfurihydrogenibium 16S rRNA[a]	515F		412	BA	GTAGCCCACATCTAGCAT	52
	907R			BA		
Toll 16S rRNA	TollF	CGCAGGCCTCTTTGTAAGTT	135	This study	CCACTCAGCACCTCTTTGTA	This study
	TollR	ATGATCTAACGGGACCATCG		This study		
aclB	ATPCLF	ATACTCARGRAATCCRACAAG	240	This study	AHCAYCCTTYCGGAAAGCCAA	This study
	aclBR			12		

[a] See Materials and Methods for an explanation of the specificity of this assay.
[b] B, primer specific for *Bacteria*; BA, primer specific for *Bacteria* and *Archaea*.

A-3′) and 907R (5′-CCGTCAATTCCTTTRAGTTT-3′) were used to amplify a 412-bp region of the 16S rRNA gene extracted from *Sulfurihydrogenibium azorense* genomic DNA (from A.-L. Reysenbach). Standard curves were created by diluting each standard over seven orders of magnitude and obtaining three replicate threshold cycle values for each dilution. DNA concentration was converted to number of gene copies using a conversion factor of 600 g of double-stranded DNA mol^{-1} $nucleotide^{-1}$. A single gene copy was assumed for all assays. Slope of the standard curves for the assays averaged −3.6, and R^2 values ranged from 0.98 to 1.00. The sensitivity of each assay was calculated from the standard curve equation for each reaction and ranged from 1 log(gene copies) to 4 log(gene copies).

Phylogenetic analysis. The 16S rRNA, *aclB*, and *nifH* electropherograms were base called using the PHRED program and assembled using PHRAP in CodonCode Aligner. All sequences of >98% similarity were clustered together in the same phylotype. The Greengenes program (18) was used to align the 16S rRNA gene sequences and find the most closely related 16S rRNA gene from cultured and uncultured bacteria. Aligned sequences were imported into the ARB program (38) and manually adjusted according to conserved regions of the gene and the established secondary structure to ensure that only homologous regions were compared. Nucleotide positions that were not conserved in more than 50% of the aligned sequences or were ambiguously aligned were masked out of the alignment so the final phylogenetic analysis was based on 1,293 nucleotides. Phylogenetic analysis was performed in PAUP* (version 40.b10; Sinauer Associates, Sunderland, MA) using parsimony, neighbor-joining, and maximum likelihood analyses. Potential long-branch attractions were investigated by adding and removing sequences across the phylogeny, especially in the *Aquificales* clades. The final 16S rRNA gene tree was created by neighbor-joining analysis with a maximum-likelihood correction using heuristic tree search with tree bisection-reconnection (TBR) branch swapping in PAUP*. The transition/transversion ratio and nucleotide frequencies were estimated according to the F84 model (21). The final 16S rRNA gene tree for the Toll sequence was created using maximum-likelihood analysis corresponding to the general time-reversible model (γ = 0.63). The starting trees were obtained by stepwise addition for both 16S rRNA gene trees. Bootstrap proportions were determined from 1,000 and 100 resamplings for the inclusive and Toll subset 16S rRNA gene trees, respectively.

All *aclB* sequences were subjected to a BLAST search to identify sequences to include in the alignment. The *aclB* alignment was based on amino acid residues and was compared to a published *aclB* alignment to ensure the correct reading frame was employed (12). Topology of the tree was explored using *Mus musculus* and *Chlorobium limicola* outgroups because the evolution of this gene is currently unclear (12, 32). The final *aclB* tree was based on 107 amino acid residues and was constructed with neighbor-joining analysis using a heuristic tree search with TBR branch swapping in PAUP*. The phylogeny of the *nifH* gene was explored through neighbor-joining analysis of amino acid and nucleotide alignments that included sequences representative of the four known *nifH* clusters (72). Trees were rooted with the *Chlorobium tepidum bchL* gene (encodes light-independent protochlorophyllide reductase), which is phylogenetically related to *nifH* sequences and has previously been used as an outgroup for *nifH* (41). The final *nifH* tree was based on 113 amino acid residues and was constructed with neighbor-joining analysis using a heuristic tree search with TBR branch-swapping in PAUP*.

16S rRNA secondary structure determination. The 16S rRNA secondary structure was determined for the Toll sequence detected in this study to ensure that its novelty was not artifactual. The 16S rRNA gene sequence was overlain onto the established structures of *A. pyrophilus*, *Thermus aquaticus*, *Deinococcus thermophilus*, and *Thermotoga maritima* obtained from the Comparative RNA Website (http://www.rna.ccbb.utexas.edu/). The structure of the hypervariable regions was determined by hand, and the molecule was checked for commonly conserved structures, compensatory changes, and long-range interactions.

Statistical analysis. A pairwise dissimilarity index F statistic (F_{ST}) was used to analyze the distribution of genetic diversity of the *aclB* sequences found in each sample using Arlequin (version 2.00; Genetics and Biometry Lab, Department of Anthropology, University of Geneva, Switzerland [http://lgb.unige.ch/arlequin/]). The forward sequences of the 333-bp *aclB* fragments were assembled into phylotypes (based on 98% similarity) by sample and aligned using Clustal W (14) in CodonCode. F_{ST} values were estimated in Arlequin for each site and were tested for significance against 1,000 randomized bootstrap resamplings.

Nucleotide sequence accession numbers. The full-length sequences determined in this study were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under the following accession numbers: EU156124 to EU156131 for *nifH* sequences, EU156131 to EU156141 for *aclB* sequences, and EU156142 to EU156157 for 16S rRNA gene sequences. The alignments used in this study are available from http://pearl3/unm.edu/site/ynp_inv_data_products.html.

RESULTS

Chemical properties and energy yield potential of sample COF_65.7. Coffee Pots is a circum-neutral to slightly acidic system that contains low concentrations of dissolved cations and anions relative to other Yellowstone thermal springs (Table 3). The low chloride and high ammonium conditions found in Coffee Pots are typical of the steam-heated, meteoric water systems found on the Mirror Plateau (23). Chemical affinities (normalized per mole of electrons transferred in the reaction) were modeled using the chemical measurements made at COF_65.7 for chemolithotrophic reactions that are frequently important in hydrothermal systems (Fig. 2). Results are presented in the same order as in previous investigations of chemical affinity (57) in Yellowstone systems. Generally, the energy available for the sequence of potential metabolic reactions is similar to that noted in Obsidian Pool, with H_2S-, H_2-, Fe^{2+}- and S^0-utilizing reactions yielding the most energy.

Phylogenetic diversity of bacteria in sample COF_65.7. The two clone libraries constructed for sample COF_65.7 (R1 and R2) contained different community compositions (Fig. 3). The R1 library was dominated by members of the *Aquificales* and *Deinococcus-Thermus*, with 58 *Thermocrinis* and 30 *Thermus* clones out of 92 total. This replicate also contained three clones of a sequence (Toll) that was approximately 99% similar

TABLE 3. Geochemical and physical measurements for sample COF_65.7

Parameter	Value
Temperature (°C)	65.7
pH	5.75
Conductivity (μS cm^{-1})	555
Chemical content (mg/liter)	
Alkalinity (as HCO_3^-)	7.8
DOC[a]	10.4
Ca^{2+}	5.6
Cl^-	0.93
K^+	10
Mg^{2+}	2.6
Na^+	27
NO_3^-	<0.1
NO_2^-	0.0033
NH_4^+	39
SO_4^{2-}	195
$S_2O_3^{2-}$	<0.3
Sulfide	0.09
Fe^{2+}	5.42

[a] DOC, dissolved organic carbon.

to BH60, a novel lineage found in Black Pool, another Yellowstone hot spring (9). All other sequences that matched Toll in a BLAST search were 85% similar or less. *Aquificales* dominated the R2 library, but *Sulfurihydrogenibium* sequences accounted for most of the clones (88 of 103). *Hydrogenobaculum* sequences (three clones) were also present. We attempted to detect *Thermocrinis* sequences from the R2 DNA sample using the *Thermocrinis*-specific primers listed in Table 2, but no amplification was detected. The remaining nine phylotypes (represented by one to three clones each) grouped within the *Alpha-*, *Beta-*, *and Gammaproteobacteria*; *Firmicutes*; *Acidobacteria*; *Deinococcus-Thermus*; and candidate division OP5. Both replicate libraries had significant *Aquificales* populations, but R1 contained only the *Aquificaceae* family (*Thermocrinis* sp.) while R2 contained representatives from the *Aquificaceae* family (*Hydrogenobaculum* sp.) and the *Hydrogenothermaceae* family (*Sulfurihydrogenibium* sp.).

16S rRNA secondary structure. The 16S rRNA secondary structure was determined for the Toll sequence by comparing it to the established structure of *A. pyrophilus*, *Thermotoga maritima*, *Thermus aquaticus*, and *Deinococcus thermophilus* to confirm that Toll's novelty was not a result of PCR artifact or sequencing error (Fig. 4). Conserved regions of the gene corresponded well to those found in *A. pyrophilus* in both sequence and structure. Most base differences throughout the structure had compensatory base differences across a stem-loop that resulted in canonical base pairing. All tertiary interactions either contained the same bases as in *A. pyrophilus* (T1, T4, T5, T6, T7, and T8) or had corresponding base changes that conserved canonical pairing (T2 and T3). Although the sequences in the V1, V3, and V4 variable regions had significantly different sequences between Toll and *A. pyrophilus*, the structures in these regions were not significantly different. Stem-loops in V2, V7, and V9 are shorter than in *A. pyrophilus*, resulting in different loop structures and a lack of bulged, nonpaired bases in the middle of stems. V2 (bases 221 to 241) is similar to the other thermophilic bacteria used for comparison, but V7 (bases 1151 to 1173) contains a much larger loop than any of the four compared structures. V9 (bases 1450 to 1476) lacks 10 paired bases in addition to a bulge of nonpaired bases, which resulted in a significantly shorter stem. However, three of the four loop bases are conserved between *A. pyrophilus* and Toll. Regions in V5 and V6 contained sequences longer than those in *A. pyrophilus*, resulting in a longer stem and larger loop in V5 and an internal loop in V6. Overall, the Toll sequence detected appears to be a nonchimeric, nonartifactual sequence with structural elements found in both the *Aquificales* and the *Thermotogales*.

Amplification of metabolic genes along the temperature gradient. Two of the seven genes that were tested, *nifH* and *aclB*, were detected by PCR amplification. The remaining five genes, *amoA*, *cbbM*, *dsrAB*, *nirK*, and *soxEF1*, failed to amplify from our samples (validated by the use of positive controls). The nitrogen fixation gene, *nifH*, was detected in samples COF_39.3 and COF_65.7, collected more than 54 m apart. Eight phylotypes were recovered with seven in COF_65.7 and one in COF_39.3. Interestingly, the most similarity (98%) was seen between phylotype F6, represented by one clone in COF_65.7, and the single phylotype found in COF_39.3. These two phylotypes plus another one grouped with the *Alphaproteobacteria*, two grouped with the *Betaproteobacteria*, and three grouped together in a clade of anaerobes (Fig. 5).

The gene for ATP citrate lyase (*aclB*) was detected in all seven samples by PCR amplification using the primers listed in Table 1. The *aclB* primers also amplified catalase genes that were similar to those found in a variety of aerobic bacteria. These sequences dominated sample COF_39.3 (65 of 77 clones) but were not amplified in any other samples. The specificity of the *aclB* forward primer and probe designed for qPCR was confirmed by comparing them with the amplified catalase genes using the NCBI bl2seq alignment tool. Ten phylotypes of the *aclB* gene were detected in the seven samples. The phylotypes were 88.3 to 100% similar at the amino acid level. The distribution of phylotypes among samples was widespread, except for phylotype G6, which was found only in sample COF_51.9. All 10 phylotypes grouped with *Sulfurihydrogenibium* species, represented by *Sulfurihydrogenibium subterraneum* in the tree (Fig. 6). *Sulfurihydrogenibium* sp. strain 153IV-9, *S. azorense*, and *Sulfurihydrogenibium yellowstonense* *aclB* sequences also grouped in this clade but were removed from the final phylogenetic analysis because they were significantly shorter (258 bp) than the *aclB* sequences obtained in this study.

qPCR quantification of 16S rRNA and *aclB* genes. *Thermocrinis* and *Sulfurihydrogenibium* 16S rRNA genes were detected by qPCR in all seven samples (Fig. 7). The abundance of *Thermocrinis* 16S rRNA genes was higher than *Sulfurihydrogenibium* 16S rRNA genes in every sample and ranged over an order of magnitude (10^7 to 10^8 gene copies per μl of DNA). *Sulfurihydrogenibium* had a wider range of gene copies, $\sim 10^{4.5}$ to $\sim 10^{6.5}$ gene copies per μl of DNA. The *aclB* gene was detected in every sample except COF_39.3. Although fluorescence was detected in this sample, exponential amplification was not observed, and the threshold cycle values were high and inconsistent, indicating that the *aclB* gene may be present in this sample at a level close to the limit of detection. The *aclB* gene copy numbers were lower than both 16S rRNA genes and ranged over an order of magnitude (10^5 to $\sim 10^{6.3}$ gene copies

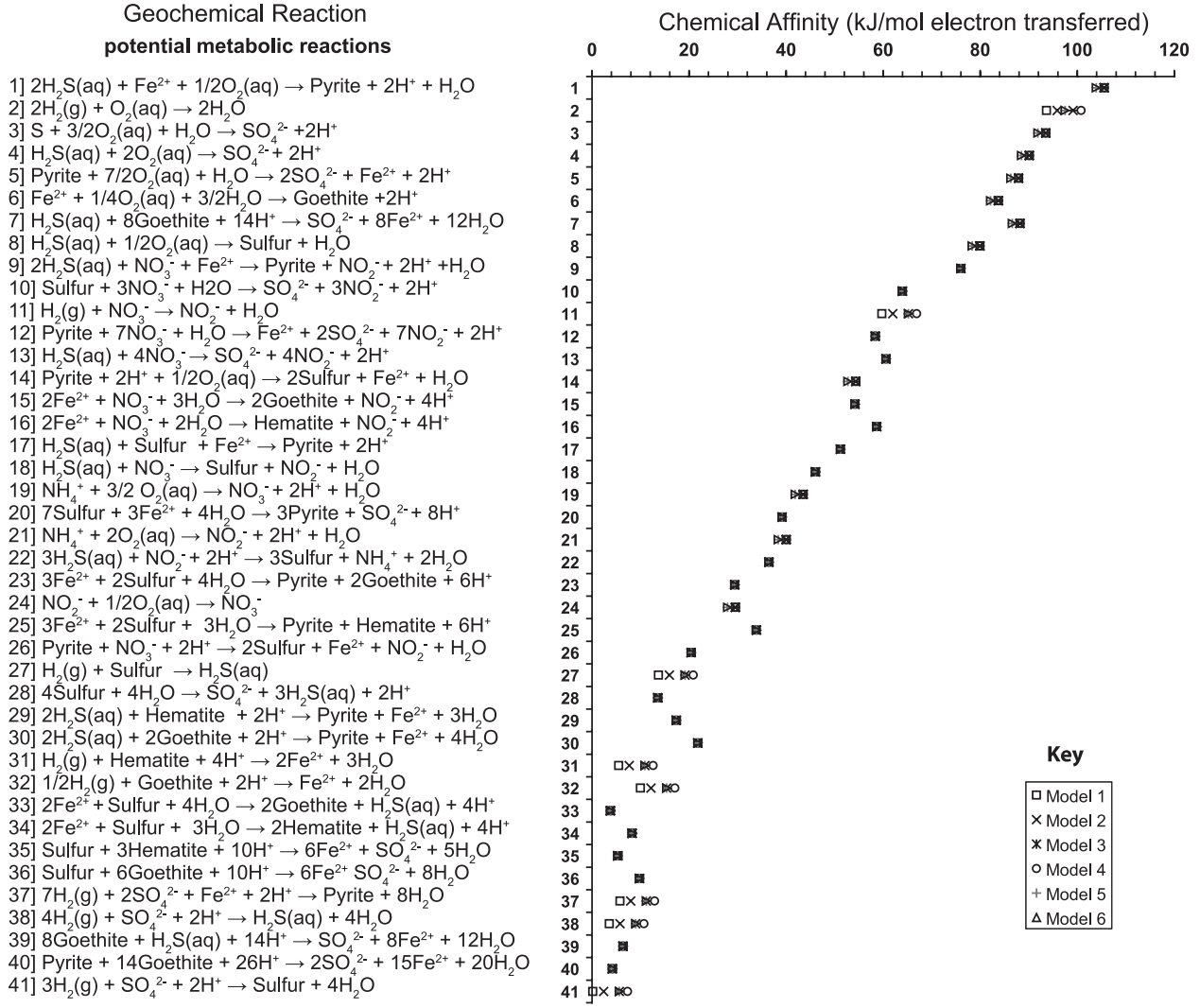

FIG. 2. Energy yield of metabolic reactions common to hydrothermal systems as a function of H_2 and O_2 concentrations reported for other Yellowstone springs (3, 57, 59). Forty-one reactions were evaluated using the analytical results for Coffee Pots (Table 3). Reactions were evaluated for a suite of six models using a range of H_2 and O_2 concentrations reported for Yellowstone hot springs: models 1 to 4 examine low (4.1×10^{-6} ppm), medium (2.05×10^{-5} ppm), medium-high (2.05×10^{-4} ppm), and high (6.67×10^{-4} ppm) H_2 concentrations, respectively, with an O_2 concentration of 0.1 ppm; and models 5, 3, and 6 examine a range of O_2 concentrations (0.01, 0.1, and 0.5 ppm, respectively) at a fixed H_2 concentration of 2.05×10^{-4} ppm. The chemical reactions are displayed and ranked in the order obtained from Obsidian Pool in a previous study (57) of decreasing energy released (per mole of electrons transferred in the reaction).

per μl of DNA). The distribution of *aclB* gene copies was closely correlated with the distribution of *Sulfurihydrogenibium* 16S rRNA gene copies along the transect ($R^2 = 0.99$), suggesting that *Sulfurihydrogenibium* species may be the source of the *aclB* genes detected along the transect.

Statistical analysis of the *aclB* sequences. Dissimilarity indices were calculated for pairs of samples, and each sample contained a significantly different ($P < 0.05$) population of *aclB* sequences (Table 4). Potential F_{ST} values ranged from 0 (variation between and within samples is equal) to 1 (all variation is between samples). The highest F_{ST} values resulted from the pairwise comparisons with COF_65.7 and COF_61.7. These samples were taken from neighboring sites (Fig. 1) and were the population maxima of *Thermocrinis* and *Sulfurihydrogenibium*, respectively, as determined by qPCR. The pairwise comparisons of the remaining five samples had similar, relatively low F_{ST} values (0.18 to 0.23).

DISCUSSION

Potential electron and energy sources in sample COF_65.7. It is important to note that our thermodynamic calculations are largely a theoretical exercise because we did not actually measure or detect oxygen and nitrate, respectively. Assuming low (detection limit) concentrations for nitrate, the modeled energetic profile of Coffee Pots showed that the processes that use oxygen, nitrate, and elemental sulfur as electron acceptors are the most energetically favorable (Fig. 2) among the 41 reactions that were run. Our results follow the general trend observed in the thermodynamic calculations for Obsidian Pool

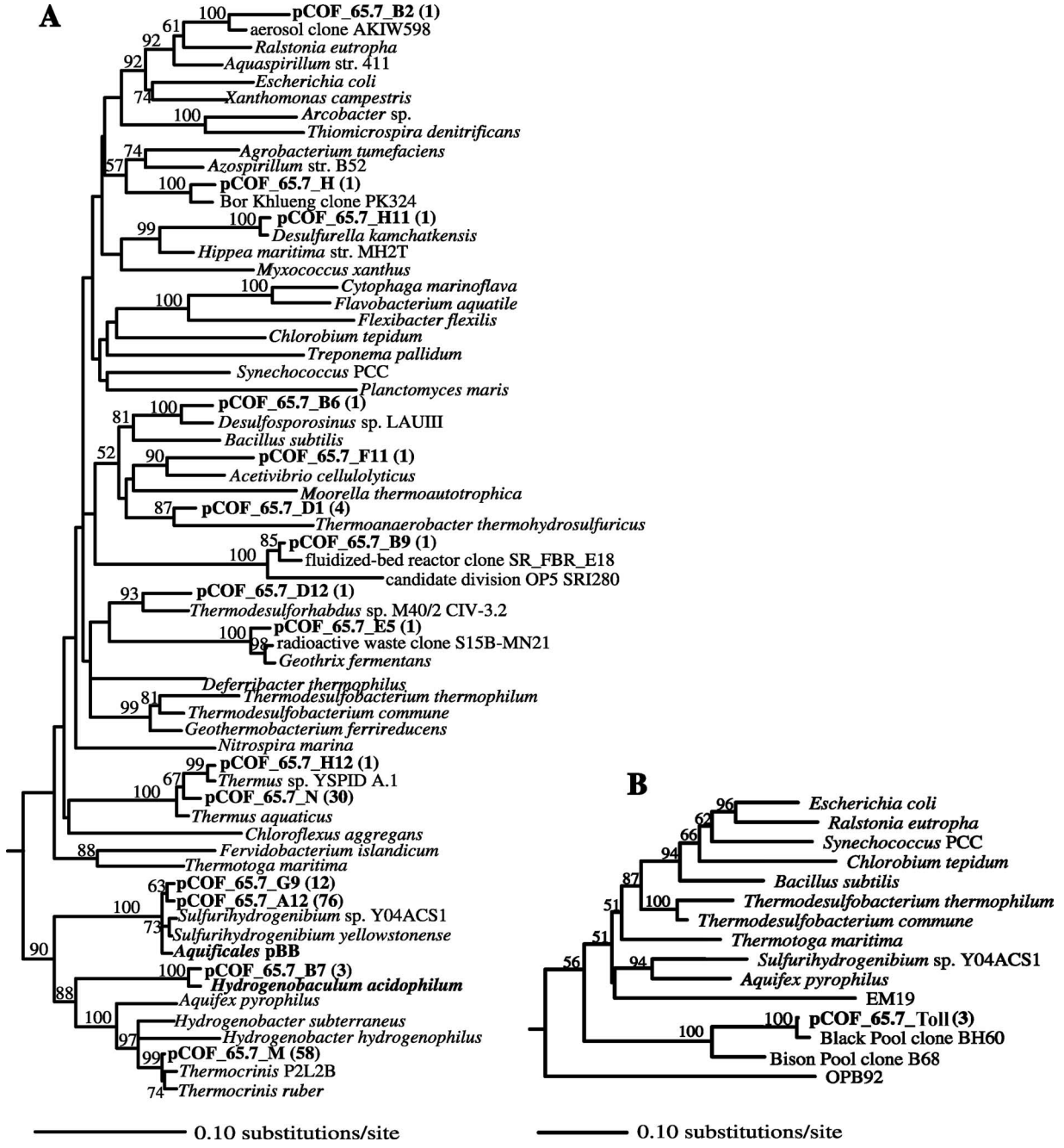

FIG. 3. (A) Phylogenetic analysis of 16S rRNA gene sequences obtained from both clone libraries for sample COF_65.7. (B) Phylogenetic tree of the Toll clone and a subset of sequences from the tree in panel A. Both trees are based on 1,293 nucleotides of the 16S rRNA gene and are rooted with *Methanocaldococcus jannaschii.* Bold names indicate sequences obtained in this study. Single-letter designations and the Toll clone represent sequences from the first clone library, and alphanumeric names represent sequences from the second clone library. The number of clones found for each phylotype is given in parentheses.

(57). It is not surprising that both systems harbor large communities of *Aquificales*, which are mostly microaerophilic or facultatively anaerobic, as hydrogen sulfide, hydrogen, and elemental sulfur oxidation were most favorable at the oxygen concentrations we used. It is interesting that within our modeled ranges, varying H_2 has the most significant effect on the ranking of the most energetically favorable reactions: at the highest values, reactions coupling H_2 oxidation to iron and sulfate reduction are favored over sulfur-utilizing reactions. Although we did not detect any bacterial *amoA* genes, the high energy yield for ammonium oxidation (Fig. 2, reactions 19 and 21) in Coffee Pots makes it possible that archaea are using this pathway (17, 25).

Diversity and phylogeny of bacteria in sample COF_65.7. In this study, we amplified a significant number of *Sulfurihydrogenibium* and *Thermocrinis* sequences from two clone libraries

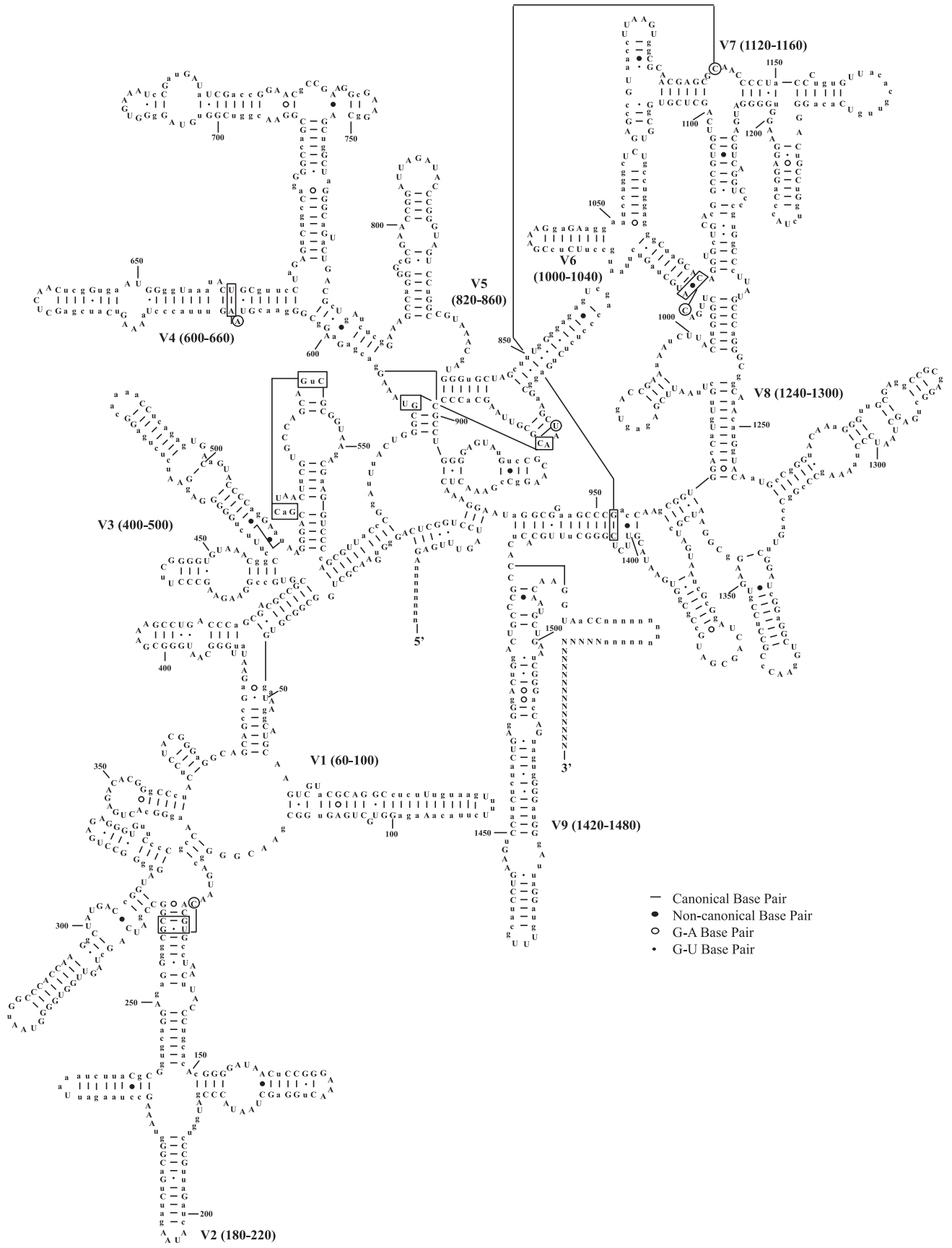

FIG. 4. 16S rRNA secondary structure for the Toll sequence. Capital letters are bases conserved between *A. pyrophilus* and Toll. Regions V1 to V9 are hypervariable regions as determined by Ashelford et al. (5); approximate nucleotide positions are given in parentheses. Regions T1 to T9 are established tertiary interactions. Numbering is unique to this structure.

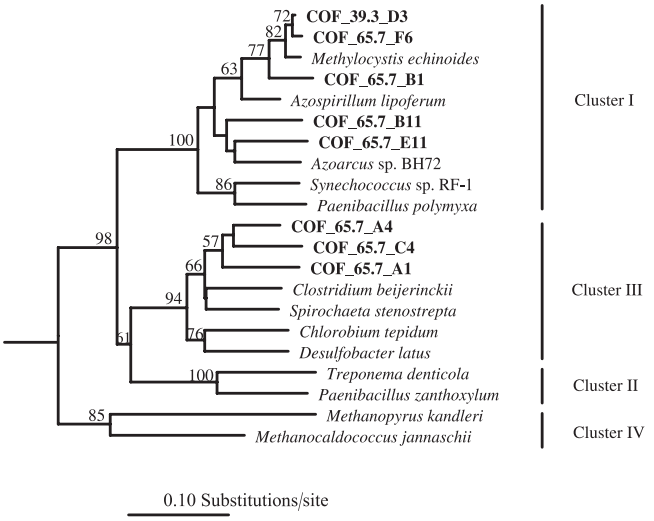

FIG. 5. Phylogenetic analysis of the *nifH* gene sequences obtained from COF_39.3 and COF_65.7. Bold text indicates sequences obtained in this study. The tree was based on 113 amino acid residues and was constructed with neighbor-joining analysis using a heuristic tree search with TBR branch swapping. The tree was rooted with the *C. tepidum bchL* gene. Bootstrap values are based on 100 resamplings.

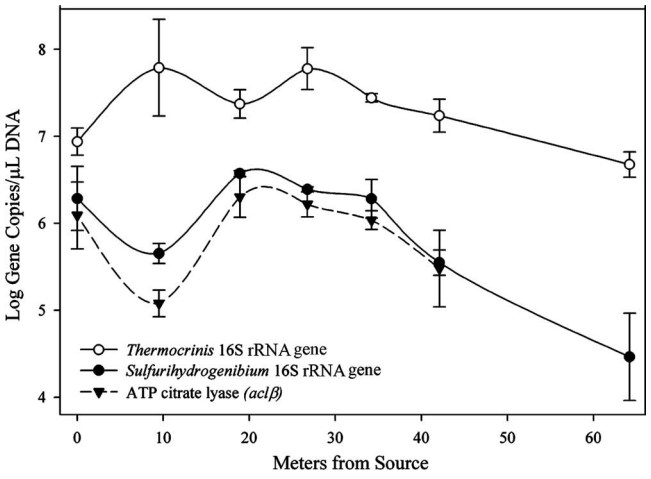

FIG. 7. Gene copy numbers for 16S rRNA and *aclB* gene assays for each sampling location. Increasing distance from the source waters of the spring correlates to decreasing temperature. Gene copy numbers were determined using assay-specific standard curves and normalized by calculating the number of gene copies per microliter of total extracted DNA in the reaction. Error bars represent the standard deviation of three replicates.

constructed from replicates of a single sample. However, neither clone library contained sequences from both of these genera, which we suspect is due to the bias of molecular techniques. The lower G+C content of the 16S rRNA gene in *Sulfurihydrogenibium* (56% for G9; 57% for A12) compared to *Thermocrinis* (61%) would favor the amplification of *Sulfurihydrogenibium* because its template would melt more efficiently (50). The predicted melting temperature of the 16S rRNA genes from the two organisms differed by 5°C. However, this does not explain why *Thermocrinis* would be detected at all, much less exclusively, when *Sulfurihydrogenibium* sequences are present in situ as well. We suspect that stochastic overamplification of one template, a nonreplicable bias that occurs in the early cycles of amplification, is responsible for this result as qPCR showed both species present at high levels in R1.

We also detected a highly divergent, phylogenetically basal sequence that we have called Toll. Comparison of this sequence to the NCBI database revealed that its closest relative (99% similar) was a partial sequence (BH60; 986 bp) from Black Pool, another Yellowstone spring. All other matches,

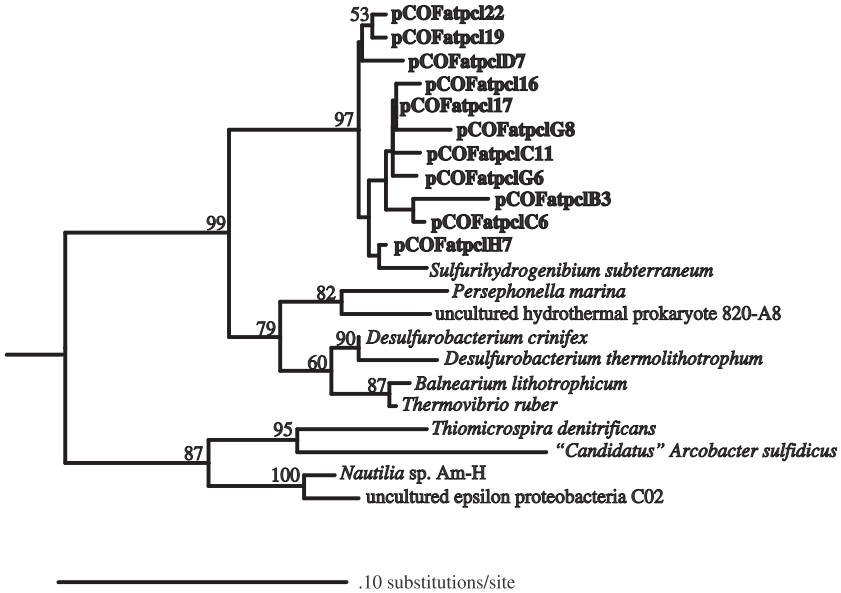

FIG. 6. Phylogenetic analysis of the *aclB* gene sequences obtained from the seven sample sites. Bold text indicates sequences obtained in this study. The tree was based on 107 amino acid residues and was constructed with neighbor-joining analysis using a heuristic tree search with TBR branch swapping. The tree was rooted with *C. limicola*. Bootstrap values are based on 1,000 resamplings.

TABLE 4. F_{ST} values for significant pairwise comparisons of the 333-bp *aclB* sequences found in each sample[a]

Sample	F_{ST} value of paired *aclB* sequences						
	COF_39.3	COF_51.4	COF_51.9	COF_57.7	COF_61.7	COF_65.7	COF_74.1
COF_39.3							
COF_51.4	0.23						
COF_51.9	0.21	0.21					
COF_57.7	0.20	0.19	0.18				
COF_61.7	0.51	0.42	0.43	0.39			
COF_65.7	0.45	0.38	0.37	0.34	0.60		
COF_74.1	0.22	0.22	0.20	0.19	0.42	0.37	

[a] Significance was determined as a P value of <0.05.

including another novel sequence from Black Pool (BH1), were 85% similar or less. Phylogenetic analysis grouped Toll with other highly divergent sequences in the most basal clade. This clade is well supported with bootstrap values of 100 at each of the nodes (Fig. 3B). However, the outer nodes of the basal lineages had lower bootstrap values as EM19 and OPB92, sequences from two candidate divisions, were frequently drawn into the Toll clade, together and separately. Additionally, Toll consistently grouped between the *Thermotogae* and *Thermodesulfobacteria* clades when 20 to 40% filters were used on all of the sequences and when EM19, BH60, BP-B68, and OPB92 were removed from the analysis, regardless of the filter used. We suspect the observed phylogenetic relatedness of these sequences is due to long-branch attractions and that their exact phylogenetic placement will remain uncertain until more sequences from these divisions are obtained.

The sequences in the Toll clade belong to organisms that seem to be minor, but persistent, constituents in Yellowstone hot springs. Although we detected only three Toll clones (3.3% of library; below detection by the 16S rRNA qPCR assay), the closest relatives of Toll also have low frequencies and accounted for only 0.8 to 8.2% of the sequences detected in their respective springs (9, 31, 42, 53). Additionally, we have detected 6 clones (of 96 total) of a sequence that is <1% different from Toll in Bechler Hot Springs, a spring more than 60 km from Coffee Pots. It is unlikely that these sequences represent transitory populations as they were collected from six springs over a decade. In addition, Toll is likely not a chimera as it formed a realistic secondary structure that was similar in structure to cultured organisms phylogenetically related to Toll. These results suggest that Toll and related sequences belong to actual thermophilic organisms with unknown in situ functions.

We also detected 11 phylotypes that grouped within eight phyla. B2, B6, F11, and E5 grouped with mesophilic genera (*Aquaspirillum*, *Desulfosporosinus*, *Acetivibrio*, and *Geothrix*, respectively) and were likely washed in from soil and groundwater, habitats from which they are commonly isolated (15, 34, 48, 51). The remaining seven phylotypes grouped with genera that grow above 40°C and are likely functional in Coffee Pots. Phylotypes D1, N, and H12 grouped with thermophilic heterotrophs found in a variety of thermal areas. The latter two grouped with *Thermus*, the only genus represented in both clone libraries, but they were more similar to other *Thermus* isolates than to each other. Phylotype H grouped with *Azospirillum*, a nitrogen-fixing heterotroph (67). It is possible that three of the *nifH* phylotypes originate from this organism as they grouped in the *Azospirillum* clade within the *Alphaproteobacteria*. Phylotypes H11 and D12 grouped with members of the *Gammaproteobacteria* that are capable of reducing elemental sulfur or sulfate and sulfite, respectively (6, 43). The latter organism may not be a numerically significant member of the Coffee Pots community as we did not detect the gene for dissimilatory sulfite reductase (*dsrAB*), an enzyme involved in both sulfate and sulfite reduction. Phylotype B9 grouped with sequences from candidate division OP5 and likely represents a novel organism.

Metabolic genes. The *nifH* gene was detected at 39.3 and 65.7°C. This gene has previously been shown to group into four clusters loosely based on 16S rRNA gene phylogeny (72). The single phylotype detected at 39.3°C grouped with the *Alphaproteobacteria* sequences of cluster I. The functionality of nitrogenases from this cluster has been well documented in a wide range of environments, and it is likely that the *nifH* sequence we detected here also represents a functional enzyme. The seven *nifH* phylotypes detected at 65.7°C are above the temperature limit (64°C) of known bacterial diazotrophs (8, 60) but lower than the recent report of archaeal diazotrophy at 92°C (40). These sequences are likely bacterial as phylogenetic analysis placed them within the *Alpha-* and *Betaproteobacteria* of cluster I and the anaerobic bacteria of cluster III, which also contains functional enzymes from known bacterial diazotrophs. Although *nifH* sequences detected in the environment are not always expressed in situ (72), the presence of these sequences suggests high-temperature bacterial diazotrophy is worthy of further investigation.

The *aclB* gene was detected in every sample by PCR, and all 10 phylotypes recovered grouped with *Sulfurihydrogenibium*. The phylogenetic reconstruction of the *aclB* sequences is consistent with the recently proposed evolution of the rTCA cycle. Initially, the rTCA cycle was thought to be operational in all species of the *Aquificales* as *acl* genes had been amplified from the *Hydrogenothermaceae* family (22) and activity had been demonstrated in the *Aquificaceae* family (7, 56). However, the *Aquificaceae* have recently been shown to utilize two enzymes, citryl coenzyme A synthetase and citryl coenzyme A lyase, to cleave citrate to oxaloacetate in place of ATP citrate lyase (32). Therefore, it is not surprising that the phylogeny and quantification of the *aclB* gene point to *Sulfurihydrogenibium* as the source of these sequences. The derived positions of these sequences in relation to *Epsilonproteobacteria* sequences is consistent with another phylogenetic reconstruction (11) and the

proposed acquisition of the *acl* gene by lateral gene transfer (32). The overall topology of the *aclB* tree did not change regardless of whether *C. limicola* or *M. musculus* was used as the outgroup, even though prokaryotic and mammalian enzymes function in different pathways and have separate evolutionary histories (4, 33).

We did not detect the *cbbM* gene, which encodes the RuBisCO (ribulose 1,5-bisphosphate carboxylase/oxygenase) form predominantly used by anaerobic bacteria, in any samples. Although the primers we used may not amplify all *cbbM* genes (20), this result is supported by the lack of visible photosynthetic pigments at the spring and the absence of 16S rRNA gene sequences from photosynthetic organisms in the clone libraries. Previous studies have shown that the Calvin cycle contributes little to bacterial biomass in other *Aquificales*-dominated springs (W. Zhao, C. S. Romanek, E. A. Burgess, J. Wiegel, G. Mills, C. L. Zhang, presented at the American Geophysical Union Fall Meeting, San Francisco, CA, 11 to 15 December 2006) and that Calvin cycle genes are less abundant than rTCA cycle genes in hydrothermal vents (11). Though all of these environments approach the temperature limit for photosynthesis (75°C) (54), competitive exclusion of photosynthetic organisms may explain the lack of Calvin cycle genes.

Quantification of *Aquificales* phylotypes and the *aclB* gene throughout the spring. *Sulfurihydrogenibium* and *Thermocrinis* have been found as dominant members of high-temperature, near-neutral springs worldwide, but they are rarely found together in the same spring (52); so the high numbers of both species throughout Coffee Pots is remarkable. Given the low carbonate in Coffee Pots spring, it is interesting that *Thermocrinis* was present in every sample because in culture its potential rTCA cycle enzyme activities are among the lowest measured (32). However, *Thermocrinis* can gain energy and carbon from formate oxidation and feed the CO_2 produced into the rTCA cycle (28, 32), allowing carbon to be metabolized heterotrophically and autotrophically simultaneously. *Sulfurihydrogenibium* species can use a variety of organic molecules (other than formate) as carbon sources (45), but they gain energy only from inorganic compounds. Although we did not test for metabolic activities, we believe this exploitation of different energy and carbon sources is a factor in the distribution of these species that is worthy of further investigation. Quantification of *Thermocrinis* and *Sulfurihydrogenibium* 16S rRNA genes showed that they each had variable populations throughout Coffee Pots. We suspect this can be partially accounted for by temperature as *Thermocrinis* species have a wider temperature growth range and higher optimal growth temperature than any *Sulfurihydrogenibium* species. However, the F_{ST} values for the *aclB* gene showed that the sequences were significantly different at every sampling point and are on the same order of magnitude for *Sulfurihydrogenibium* 16S rRNA gene sequences found throughout Yellowstone (63). This level of divergence in a single hot spring suggests that *Sulfurihydrogenibium* organisms may not be phenotypically or genotypically identical throughout Coffee Pots.

A unified description of microbial spatial patterns has been difficult to construct, especially for thermophilic bacteria and archaea. Some studies have found evidence of locally adapted thermophile populations but have been unable to link their distribution to temperature, alkalinity, or chemical composition (47, 71). Similar problems have arisen in attempts to correlate metabolic capabilities of individual microbes or entire ecosystems to chemical and physical parameters even though many thermophiles require inorganic substrates for energy generation. We have shown that the energy available for different metabolic processes varies greatly in the water overlying just one sample. However, the small size and limited mobility of prokaryotes make it likely that their distribution is affected on an even smaller scale (micrometer to centimeter), especially when communities are fixed in place through filament formation. In this study, we found evidence of significantly different *aclB* sequences throughout the thermal transect that correspond to spatial variation in the *Sulfurihydrogenibium* population, which is likely a function of variable microscale conditions. Identifying locally adapted ecotypes and evaluating the genetic and physical parameters that explain their distribution could advance further metabolic characterization of the *Aquificales*.

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