A Test for Airborne Dispersal of Thermophilic Bacteria from Hot Springs

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ABSTRACT

Physical and chemical barriers separate individual terrestrial hot springs and their affiliated communities of thermophilic bacteria. However, 16S rRNA gene sequence comparisons suggest that seemingly identical or closely related bacterial species are found in multiple hot springs that are separated by distances that range from a few meters to thousands of kilometers. To investigate whether this dispersal could result from airborne transport of bacteria on water vapor, steam was collected from multiple sites at approximately 1 meter above the flowing hot water of Spring AT-1, which is part of Angel Terrace in the Mammoth Hot Springs complex of Yellowstone National Park. The trapped steam was analyzed for the presence of bacterial 16s rRNA gene sequences and these were directly compared with sequences identified in Spring AT-1 itself. Of the 31 different sequences identified in the steam: 1 sequence is 100% identical to a sequence previously identified in Spring AT-1; 2 sequences are similar to Spring AT-1 sequences; and 3 more sequences are affiliated with thermophilic bacteria not observed in Spring AT-1. These findings indicate that that thermophilic bacteria are present in the steam, and suggest that aerosolization of hot spring thermophiles is a necessary first step for airborne transport.

Key Words

airborne transport
bacterial dispersal
colonization
Mammoth Hot Springs
thermophile

1.0 INTRODUCTION

Bacteria have evolved to inhabit specific niches in the environment, with some species thriving in extremes of temperature, salinity, and pH. Although microbiologists are no longer surprised to discover so called "extremophiles" inhabiting seemingly inhospitable environments (Pace 1997), little or nothing is known about how organisms with such specific environmental requirements are dispersed and able to colonize new and suitable locations. Microbes living in thermal locations such as deep-sea hydrothermal vents and terrestrial hot springs, for example, are faced with growth-inhibiting, low temperature barriers to expansion. Terrestrial hot spring communities are separated by a dry and cool landscape at the earth's surface. While subsurface hydrothermal connections may exist between hot springs in close proximity to each other, even springs within a particular geothermal region such as Yellowstone National Park (YNP) may be hydraulically isolated from each other (White et al. 1988; Sorey 1991; Kharaka et al. 1992; Mariner et al. 1992; Rye and Truesdell 1992). The absence of a universal network of subsurface hydrothermal fluid connecting all of the world's thermal features precludes the possibility of a global conveyor belt to distribute thermophiles.

The adverse environment surrounding an extremophile community will prevent microbial motility (i.e., self-propulsion). Motility requires an expenditure of energy that, for example, a thermophile adapted to life at 80°C could not sustain after entering into a 4°C or even 30°C surroundings. Additionally, many thermophiles, lacking flagella, are simply not capable of self-propulsion or not observed to exhibit gliding motility (Overmann 2000; Hanada and Pierson 2002; Huber and Eder 2002; Huber and Hannig 2003).

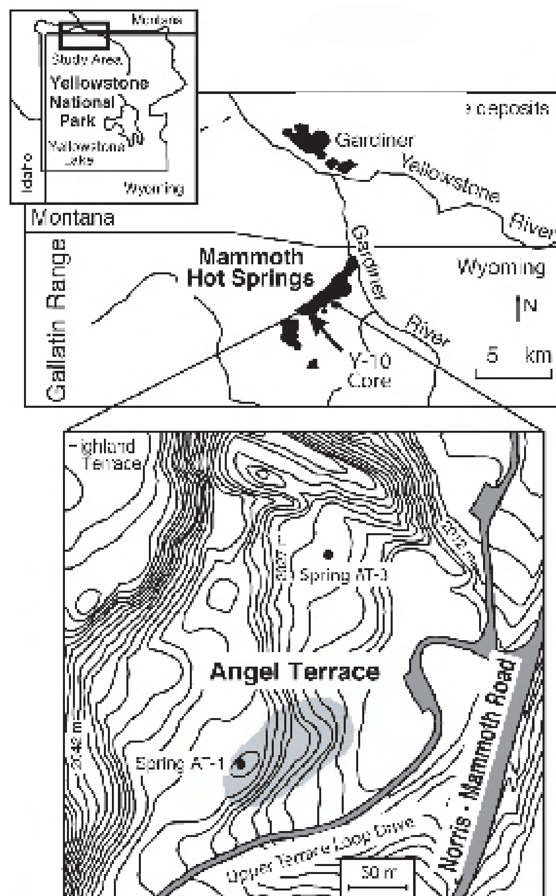

Figure 1A. Map showing the location of Spring AT-1, the study site at Angel Terrace, which is part of the larger Mammoth Hot Springs complex in Yellowstone National Park.

Figure 1B. Photograph of Angel Terrace taken from Upper Terrace Loop Drive, facing west (March 2001). The vent of Spring AT-1 is located at the top of the first ridge, on the center-left side of the photograph, and is not visible in this picture. Water erupting from Spring AT-1 flows down the slope face (proximal slope facies), primarily along the forest line. Some snow is visible in the foreground, but the white of the slope face is travertine.

Figure 2. Photograph showing terraced travertine deposits at Spring AT-1, facing south (May 2001).

Nonetheless, environmental barriers to motility do not appear to be absolute barriers to dispersal. Very similar 16S rRNA gene sequences attributed to Aquificales, for example, have been identified from hydrothermal sources in several globally distributed locations (Hugenholtz et al. 1998; Reysenbach et al. 2000; Takacs et al. 2001; Yamamoto et al. 2002; Fouke et al. 2003). Most recently, a survey of 16S rRNA gene sequences found in Spring AT-1 of the Mammoth Hot Springs complex identified several thermophile-affiliated sequences that share a high degree of similarity to sequences found in other springs in Yellowstone and from springs located in other states or continents (Fouke et al. 2003). The geographic isolation of the individual springs indicates that dispersal is most likely the consequence of passive transport of the bacteria as opposed to active motility.

For this study, an airborne transport model was deemed to be the most plausible explanation for both global (thousands of kilometers) and regional (tens to hundreds of kilometers) transport (Ehrlich et al. 1970; Griffin et al. 2002; Pósfai et al. 2003). To test this theory, steam was collected from the area surrounding Spring AT-1 at Mammoth Hot Springs. The steam was tested for the presence of 16S rRNA gene sequences, and then these sequences were compared with the existing (Fouke et al. 2003) database of sequences found in Spring AT-1. In this manner, airborne thermophilic organisms might be directly linked to a point source.

Figure 3. Photograph of the transition between the apron and channel facies and the pond facies, showing both biotic and abiotic solid phase travertine deposition (March 2001). The elongated white structures are aragonite-encrusted filaments of Aquificales spp. that extend from the apron and channel into the pond. In the pond, other bacteria colonize these structures, leading to a tan-green color; the filaments grow thicker with layers of microbial growth and aragonite and calcite precipitates. Within the pond are complex mat-forming microbial communities. Gas produced by these communities causes the mats to lift upward, forming peaks or pillars, which eventually become calcified with a mix of aragonite and calcite (the tan-green balls).

2.0 MATERIALS AND METHODS

2.1 Geological Setting

Spring AT-1 is located on Angel Terrace, in the upper terrace region of the Mammoth Hot Springs complex (**Figure 1, previous page**). Subsurface water erupting at Spring AT-1 has a temperature range of 70-73°C, but cools to 25°C by the time it flows 50 m. Within this distance CO_2(aq) degases, pH increases from 6 to 8, and travertine precipitates at rates exceeding 3 mm/day (Sorey 1991; Ford and Pedley 1996; Fouke et al. 2000, 2003; **Figure 2**). The Spring AT-1 drainage outflow has been subdivided into a series of ecological partitions along the continuous flow pathway of the spring water (Fouke et al. 2000). These sub-environments, called sedimentary depositionalfacies, are based on changes in the shape and chemical composition of the travertine that is precipitated on the floor of the spring outflow channel. Travertine composition changes from nearly 100% aragonite to nearly 100% calcite with increasing distance from the vent, and exhibits a great variety of macro- and microscopic changes in crystal growth shape

and form (Fouke et al. 2000, 2003). The dynamic interplay between fluid flow and precipitation (and possible biotic influence) is complex and not yet understood (**Figure 3**). Ongoing surveys of the microbial communities inhabiting Spring-AT1 have identified 16S rRNA gene sequences affiliated with at least 21 divisions of bacteria (Fouke et al. 2003; Zhang et al. 2004).

2.2 Steam Collection

Steam samples were collected on sheets of aluminum foil that were prepared in a controlled laboratory setting to remove or destroy all cells and nucleic acids. The sheets of foil were treated with a wash of 50% bleach followed by a rinse with PCR-quality (sterile, nucleotide, and nuclease-free) water. The foil was then folded into a triangular funnel shape with 15-pound test fishing line folded into one side (to be used to tie the foil to supports in the field). The funnel sheets of foil were rewashed with bleach, rinsed, exposed to sanitizing levels of ultraviolet light, and then packaged within an additional treated sheet of foil. This foil package was autoclaved and then enclosed in a nucleotide and nuclease-free ziplock bag for transport to and from the field site. Several funnels were prepared, including some used as negative controls.

At the field site, the foil funnels were mounted between two poles ~1 m above the ground or water surface either directly over or adjacent to various locations within the drainage path of Spring AT-1 (**Figure 4**). On some of the foil funnels, a sterile 50 ml disposable polypropylene centrifuge tube was attached to collect additional steam condensate. The tube was attached by applying duct tape to the outside of the tube and to the back (not used for steam collection) side of the foil. The bottom corner of the foil extended into the tube to direct condensate into the tube, and both the tube and tape were attached without contacting the collection face. Extreme care was taken not to contaminate the collection devices: latex gloves were worn and washed with a 50% bleach solution whenever the foil collection devices were handled. A negative control was unpacked in the field and attached to a collection tube, and then the entire unit was repackaged to determine if field manipulation might contribute to contamination. The experimental exposures lasted for either 48 or 72 hours while steam condensed upon the surface of the foil. After exposure, the triangles were repackaged in their sterile foil wraps and bags, and were transported on ice to the Fouke Geomicrobiology Laboratory at the University of Illinois.

Figure 4. Photograph showing one of the triangular foil sheets used to collect steam at the apron and channel facies of Spring AT-1 (November 2002).

2.3 DNA Extraction And Polymerase Chain Reaction (PCR) Assays

Working in a clean laminar flow hood, 1 ml of PCR-quality water (DNA, RNA, DNAse, and RNAse free) was used to wash the surface of each foil funnel. Negative controls were included in the analysis and were treated identically. The wash water was divided into 3 aliquots, and each underwent a different DNA extraction protocol—freeze-thaw, chemical, or physical (bead beating) lysis. The extraction and PCR amplification of 16S rRNA gene sequence protocols are essentially the same as previously described (Frias-Lopez et al. 2002; Fouke et al. 2003). PCR analysis of negative controls carried into the field and back revealed no evidence of contamination.

2.4 DNA Sequencing And Sequence Analysis

The PCR-amplified 16S rRNA gene products were purified using the Wizard® PCR Prep kit (Promega, Madison WI) and cloned into the pGEM®-T cloning vector (Promega). Unique clones were selected for sequencing using a restriction fragment length polymorphism (RFLP) protocol previously described (Fouke et al. 2003). Partial sequencing of the cloned rRNA genes was performed by the W. M. Keck Center for Comparative and Functional

Table 1. **Examples of sequences isolated from Spring AT-1 that are closely related to sequences from other thermal locations.**

Accession Number of AT-1 sequences	Division	% Identity	GenBank Accession Number and Description of Closest Relative	Location*:
AF445689	Beta Proteobacteria	98% 97%	AF026979, Unidentified beta proteobacteria OPB30 AF542381, Unidentified beta proteobacteria, clone CD20H04	Obsidian Pool, MVA Wasswick Spring, Idaho
AF445699	Beta Proteobacteria	99% 96% 96%	U46749, beta proteobacteria OS-ac-15 AF309815, uncultured synthetic wastewater bacterium tmbr15-22 AF125877, dehydroabietic acid-degrading bacterium DhA-73	Octopus Spring, LGB Bioreactor in Indiana Thermal site, Vancouver, BC
AF446288	Bacteroides, Cytophaga, Flexibacter Group	99% 94%	AF027008, Unidentified Cytophagales OPB73 AY555776, Uncultured bacterium clone PK10	Obsidian Pool, MVA Bor Khluenghot spring in Thailand
AF445735	Green non-sulfur bacteria	99%	AF02703 Unidentified green non-sulfur bacterium OPB65	Obsidian Pool, MVA
AF445722	Cyanobacteria	99% 98% 97%	M62776, Uncultured cyanobacterium OS Type B AF285243, *Synechococcus* sp. OH32 AY145685, Unidentified cyanobacteria, clone TS4E06	Octopus Spring, LGB Oregon Trinity Springs, Idaho
AF445745	Firmicutes	97%	L04707, Eubacterium sp. (OS type L)	Octopus Spring, LGB
AF445687	OP11	98%	AF027030, Candidate division OP11 clone OPB92	Obsidian Pool, MVA
AF445734	Aquificales	99% 98%	AF027098, Unidentified Aquificales clone OPB13 AF453509, Uncultured Aquificales bacterium clone pTthxG5	Obsidian Pool, MVA Kamchatka
AF445739	Aquificales	99%	AF113542, Uncultured Aquificales pBB	Calcite Springs, TF
AF445644	Thermus-Deinococcus	99% 99% 98% 96%	L10070, Thermus YSPID A.1 AF027020, Unidentified Thermus OPB31 AY053486, Uncultured bacterium AT425_EubC9 Y18408, *Thermus igniterrae*, strain GE-2	Octopus Spring, LGB Obsidian Pool, MVA Gas hydrates, Gulf of Mexico Iceland

* MVA = Mud Volcano Area, Yellowstone National Park; LGB = Lower Geyser Basin, Yellowstone National Park; TF = Tower Falls, Yellowstone National Park. Information about where sequences were obtained is referenced in GenBank and can be accessed using the accession number provided with each sequence.

Genomics at the University of Illinois. Sequences were edited manually using Sequencher v. 4.2 (Gene Codes Corporation, Ann Arbor MI), and after editing, unambiguous sequence lengths ranged from 411 to 706 bp. The sequences were compared with the GenBank database to generate a list of the 100 most similar sequences using the basic local alignment search tool (BLAST), and also searched against our own library of sequences from Spring AT-1 (Altschul et al. 1990). For the purpose of this study, sequence alignments were made using Clustal X (Chenna et al. 2003), and assessments of percent identity shared between sequences were deemed as a sufficient analysis. A 100% sequence identity in the 16S rRNA gene sequence was considered an exact match between steam sequences and spring sequences. Anything less than 100% identity, while perhaps suggestive of a phylogenetic relationship, was interpreted as insufficient evidence to suggest that sequences present in steam originated from the spring.

The GenBank accession numbers for each 16S rRNA gene sequence generated in this study are listed in Table 2.

3.0 RESULTS

3.1 Evidence For Dispersal

Ongoing analysis of Spring AT-1 has identified 657 sequences representing at least 21 divisions of bacteria (Fouke et al. 2003; Zhang et al. 2004). A majority (>90%) of these sequences are found only within one of five distinct zones (sedimentary depositional facies) within the spring, allowing the sequences to be tightly correlated to environmental parameters of temperature, pH, geochemistry, and mineralogy (Fouke et al. 2003). Many of these sequences share >97% sequence identity to cloned 16S rRNA gene sequences isolated elsewhere in YNP, or from hydrothermal systems located in other states, or even other continents (**Table 1**). Several of the most closely related sequences are those shared between Spring AT-1 (**Figure 1**) at Mammoth and springs in Norris Geyser Basin, located 34 km to the south. Although the sequence comparisons do not identify the direction of transfer between springs, a source population, or when transfer occurred, the similarities do imply a common evolutionary ancestor.

3.2 Steam Collection

Foil collection devices were deployed in early June (two devices) and again in mid-March (three devices) to collect steam samples. Not surprisingly, more steam condensed on the foil during March due to the colder air temperatures. Steam on the foil funnels rapidly evaporates in the dry mountain atmosphere at Mammoth, leaving behind cells and a residue haze (presumably carbonate) on the foil surface. Each collection apparatus was installed approximately 1 to 1.5 m above the surface of the spring drainage system, and locations were selected at the top of the terrace to avoid splashing from the spring water cascading over the terracettes. The aim was to collect only the lowest-mass droplets of water found in steam, which are those that could most readily be carried long distances. Some collection devices were damaged by wind or possibly by passing animals, but three survived for analysis (1 from June, and 2 from March).

3.3 Analysis Of The Captured Bacteria

Detection of bacterial 16S rRNA gene sequences proved difficult, presumably due to low cell counts in the air and on the foil surface. Rinsate from the foil surface was Gram-stained and analyzed by light microscopy to search for cell bodies, but less than 20 putative cells were detected. Using PCR amplification for 16S rRNA genes, a total of 31 different sequences were generated (**Table 2, next page**). Of the steam sequences, only one, clone FL11A06 (GenBank accession AY145634), shares 100% sequence identity with a previously detected sequence from Spring AT-1. This sequence was found during a previous study of the spring's apron and channel facies (Fouke et al. 2003); the same facies where the steam collection device was located. The apron and channel facies is characterized by a temperature range of 65-69°C, pH ~6.5, and 1-5 cm deep spring water flowing over nearly 100% aragonite sediments (Fouke et al. 2003). Clone FL11A06 also shares 99% identity with clone FL12B02 (GenBank accession AY145647), which was found in the apron and channel facies of Spring AT-11.1, located 20 meters northeast of the vent of Spring AT-1.

Of the remaining 30 sequences, none shared 100% sequence identity with clones identified from Spring AT-1

Table 2. **Sequences isolated from steam and their closest relative sequences.**

Accession Number of Steam Sequences	Division	% Match	GenBank Accession Number and Description of Closest Relative	Location*
AY162832	Alpha Proteobacteria	95%	AB016865, *Acetobacter* sp.	No information
AY145638	Alpha Proteobacteria	95%	AY494637, Uncultured *Acidisphaera* sp. clone ALPHA7	fish gills
AY162829	Alpha Proteobacteria	96%	AY494637, Uncultured *Acidisphaera* sp. clone ALPHA7	fish gills
AY145640	Alpha Proteobacteria	95%	AJ292618, Uncultured eubacterium WD256	PCB contaminated soils
AY145648	Alpha Proteobacteria	96% 85%	X77468, *Acidomonas methanolica* AF445716, Uncultured alpha proteobacterium SM2C02	Pure culture Spring AT-1 distal slope facies
AY145649	Alpha Proteobacteria	96% 93%	AY250859, Uncultured alpha proteobacterium clone BPU225 AY140237, Iron-oxidizing acidophile Y005	McMurdo Dry Valleys, Antarctica Yellowstone NP
AY145658	Alpha Proteobacteria	99% 97%	AY250855, Uncultured alpha proteobacterium clone BPS153 AF465654, clone YNPRH71B	McMurdo Dry Valleys, Antarctica Soil thermophile from Yellowstone
AY145632	Alpha Proteobacteria	98%	X75617, *Gluconacetobacter liquefaciens*	No Information
AY145652	Alpha Proteobacteria	96%	AF127407 , *Gluconacetobacter sacchari*, isolate SRI1794	Sugar cane leaves
AY145641	Alpha Proteobacteria	95%	AF465654, Uncultured alpha proteobacterium YNPRH71B	Soil bacteria from a thermal gradient
AY162827	Alpha Proteobacteria	97%	AY100556, Uncultured bacterium, clone cvf67053	Air sample
AY162830	Alpha Proteobacteria	98%	AY100556, Uncultured bacterium, clone cvf67053	Air sample
AY145635	Alpha Proteobacteria	95%	AJ290043, Uncultured bacterium GKS2-218	Lake bacterioplankton
AY145654	Alpha Proteobacteria	96%	AJ292596, Uncultured eubacterium WD236	PCB contaminated soils
AY162828	Alpha Proteobacteria	98%	AJ292611, Uncultured eubacterium WD2108	PCB contaminated soils
AY145656	Alpha Proteobacteria	98%	AJ292611, Uncultured eubacterium WD2108	PCB contaminated soils
AY145634	Beta Proteobacteria	100% 99%	Clone FL11-A06 AY145647, Uncultured beta proteobacterium FL12B02	Spring AT-1 apron and channel facies Spring AT-11.1 apron and channel
AY145664	Gamma Proteobacteria	99%	AY211102, *Planomicrobium mcmeekinii*	Airborne desert dust in Mali, West
AY145650	Fibrobacter/ Acidobacteria	97% 94%	AY587229, Acidobacteriaceae bacterium TAA48 AF465656, clone YNPRH46A	No Information Soil thermophile from YNP
AY145655	Fibrobacter/ Acidobacteria	97%	AJ292587, Uncultured eubacterium WD277	PCB contaminated soils
AY145667	Firmicutes	96%	AB021184, *Bacillus ehimensis* (strain:IFO15659)	No Information
AY145668	Firmicutes	98%	AY211170, *Planococcus* sp. 'Mali 167'	Airborne desert dust in Mali, West Africa
AY145669	Firmicutes	95%	AF010057, Unidentified eubacterium	Soil

Table 2. **Sequences isolated from steam and their closest relative sequences, continued**

Accession Number of Steam Sequences	Division	% Match	GenBank Accession Number and Description of Closest Relative	Location[a]
AY145670	Firmicutes	98%	AF479357, Glacial ice bacterium G50-TS4	Glacial or subglacial environments
AY145665	BCF	95% 92%	AF392705, Uncultured bacterium clone CEA1 AF446290, Uncultured CFB group bacterium FL14C11	No Information Spring AT-1 pond facies
AY145666	BCF	95%	AJ318172, Uncultured bacterium, clone BIsi1	No Information
AY145660	Eukaryote; chloroplast	90% <85%	Y17632, *Closteriopsis acicularis* plastid AF445656, Uncultured eukaryote clone SM1B08	No Information Spring AT-1 proximal slope facies
AY145653	Eukaryote; chloroplast	99%	D17510, *Pinus thunbergii*, chloroplast	Pure culture
AY145659	Eukaryote, mitochondria	95%	Z14059, *Secale cereale* mitochondrion	Pure culture
AY145633	Unknown	97% 94%	AJ292591, Uncultured eubacterium WD208 AF445712, Uncultured alpha proteobacterium SM2B06	PCB contaminated soils Spring AT-1 proximal slope facies
AY145636	Unknown	91%	AF316711, Uncultured Crater Lake bacterium CL120-78	lake bacterioplankton

*Inferred from the publication in which the sequence was first published or the GenBank submission content; further information may be retrieved using the GenBank accession number.

water. The steam clones were also compared with the entire non-redundant nucleotide sequence database in GenBank. However, BLAST analysis did not find any 100% identical sequence matches with previously identified sequences. One sequence, clone FL10E11, was most similar to a cloned sequence obtained from soil along a thermal gradient in another region of Yellowstone. This clone, uncultured alpha proteobacterium YNPRH71B (AF465654), shares 95% sequence identity to the steam clone. Two other clones from the steam share significant similarity with sequences found in Spring AT-1; however, the most similar matches from GenBank are not sequences reported as having been isolated from thermal features. These two steam sequences include clone CD21F11, which shares 92% sequence identity with clone FL14C11 (GenBank accession AF446290), previously isolated from the pond facies. The pond facies is characterized by a mixture of roughly 80% aragonite and 20% calcite, temperatures (for the sample from which this sequence was isolated) of 45-55° C, and a pH of 7.4 (Fouke et al. 2003). The second clone is FL10C05; it shares 94% sequence identity with clone SM2B06 (GenBank accession AF445712). This sequence was identified in the proximal slope facies. The proximal slope facies is characterized by a decreasing ratio of aragonite to calcite as the spring water travels further from the vent, a broad temperature range of 35-55° C, and a pH of ~7.7, also depending upon the distance from the vent (Fouke et al. 2000, 2003).

Two other sequences share very limited similarity with sequences found in Spring AT-1. Clone FL12C01 shares only 85% sequence identity to clone SM2C02 from the distal slope facies and clone FL12E12 shares less than 85% sequence identity with clone SM1B08. Additionally, three other sequences share significant similarity with sequences identified from other YNP thermal features. Clone FL12E03 shares 97% sequence identity with clone YNPRH71B (GenBank accession AF465654) and clone FL12C07 shares 94% sequence identity with YNPRH46A (AF465656). Both of the YNPRH clones are sequences isolated from soil

along a geothermal gradient in YNP. Ironically, the closest matching sequence (99% sequence identity) to FL12E03 in GenBank is a cloned sequence from sediments collected from the McMurdo Dry Valleys, Antarctica. The third sequence, clone FL12C05, shares 93% identity with an iron-oxidizing acidophile clone isolated in Yellowstone, Y005 (AY140237). The remaining sequences have not been previously identified in hot spring AT-1, or in any other thermal feature.

Any 16S rRNA gene sequences from steam that exhibited less than 100% sequence identity to Spring AT-1 sequences cannot be definitively interpreted as having originated from the spring. Steam sequences sharing 90-99% sequence identity with an AT-1 spring clone or other thermophile are mentioned here only to the extent that the relationship may be suggestive of a thermophilic source. It must also be noted that the clone library for Spring AT-1 is not exhaustive, and that statistical analyses suggest that there are many more as yet unidentified sequences to be found (Fouke et al. 2003).

4.0 DISCUSSION

4.1 Geological Time Constraints

Current estimates have calculated the average rate of change in a 16S rRNA gene sequence to be 1-2%/50 million years (Ochman and Wilson 1987; Moran et al. 1993; Ochman et al. 1999). This calculation for a "molecular clock" remains controversial, in part due to the unknown impacts of environmental stress, population size and isolation, and the consistency of this mutation rate between different species or divisions of bacteria (Ochman et al. 1999). Estimates of the age of hydrothermal features within YNP are constrained by several geological events. Analysis of the Y-10 sample core drilled at Mammoth Hot Springs has determined that the present travertine deposits date back 7700 +/- 440 years (White et al. 1975; Sturchio 1990). The springs themselves may have existed for a longer period; however, the Pinedale glaciation (70,000-13,000 ka) erased much of the sedimentary evidence (Sturchio 1990; Chafetz and Guidry 2003), and the spring's activity during the Pinedale remains unclear. Samples collected at the base of the travertine deposits found at Terrace Mountain, located 1-2 km southwest of the Y-10 drill site, provide Uranium-series ages of 389,000 +/- 26,000 and 361,000 +/- 23,000 years. These deposits are believed to have been formed at the bottom of a lake, but may indicate that spring activity dates back nearly 400,000 years (Bargar 1978; Chafetz and Guidry 2003). Thermal activity in Yellowstone dates back to at least ~2.1 Ma, when the first major eruption occurred and created the Huckleberry Ridge Tuff deposits (Gansecki et al. 1998; Bindeman et al. 2001). Volcanism has occurred sporadically in Yellowstone since the Pliocene, with the last period lasting from 150,000 to 75,000 ka (Bindeman et al. 2001). These dates would constrain mutation and speciation of thermophiles within the entire Yellowstone region to a period of less than 2.2 million years.

In YNP's Mammoth area, hot springs periodically seal themselves off with their high rates of precipitation (more than 1.5 m/year; Fouke et al. 2000; Fouke et al. 2003; Bonheyo *submitted*), and new springs emerge sporadically around the complex. The lifespan of an individual spring vent is estimated to range from days or weeks to several years. Springs AT-1.1 and AT-1.3, which first erupted in January 2000, flowed for more than 2 years before drying-up sometime between July and November, 2002 (Friedman 1970; Bargar 1978; Sorey and Colvard 1997; Bonheyo *unpublished*; NPS *personal communications*). The eruption of a new hot spring creates an environment suitable for colonization by thermophilic bacteria but little is known about how thermophiles reach a new environment or how widely separated the source populations can be. Additionally, there exists little or no understanding about the potential for, or extent of, mixing between isolated thermophilic communities.

4.2 Source Populations

There are two likely explanations for nearly identical bacterial gene sequences to be found in multiple locations. Either the population at one site was the source for the population of the second location, or both of these locations were "seeded" by yet another source population. For sequences belonging to a lineage such as Aquificales, in which almost all known representative species are thermophilic (Huber and Eder 2002), the transported cells

must have been thermophiles. A third possibility, that two distinct species underwent convergent evolution to have similar 16S rRNA gene sequences while also adapting to become thermophilic, is highly improbable (Asai et al. 1999; Kurland et al. 2003).

4.3 Possible Methods Of Transport

Based upon careful alignments, several of the sequences sharing the greatest similarity to sequences from Spring AT-1 (**Figure 1**) were those identified from springs in the Mud Volcano Area, located approximately 50 km southeast of Mammoth (**Table 1**). Theoretically, localized transport could occur via delivery by a fluid medium—spring runoff or shared underground plumbing for example. However, the possibility of fluid transport between Mammoth and the Mud Volcano Area is unlikely. The distance and multiple altitude changes separating the springs prevent the surface waters of one spring from flowing directly into the other. The Mammoth Hot Springs and Mud Volcano areas are geographically and geologically separated, with Mammoth lying to the north of the Yellowstone caldera and Mud Volcano within the caldera ring-fracture system (Fournier et al. 1992). Mammoth and Mud Volcano, as well as the other thermal areas in Yellowstone, are hydrologically recharged at shallow as well as deep levels in the subsurface (Rye and Truesdell 1992, 1993). Shallow recharge is derived from local rainfall and associated runoff. Conversely, deep groundwater at Mammoth, and potentially at Mud Volcano, is recharged in the western part of the Yellowstone caldera. Elemental and isotopic analyses of the spring waters suggest that this deep recharge is a combination of magmatic water and meteoric water derived from the Gallatin Range that flows in the subsurface along major North-South faults (White et al. 1988; Sorey 1991; Kharaka et al. 1992; Mariner et al. 2002; Rye and Truesdell 1992, 1993). However, the separate shallow recharge zones and < 350oC temperature of the deep recharge levels (Fournier 1989) indicate that there is little possibility of a common microbial community that is dispersed via underground conduits to both locations.

Localized transport by animal or insect vector is another intriguing possibility. Many animals including elk and bison visit the springs for warmth in winter (National Park Service 2004; NPS *personal communications*), and these animals may travel several kilometers, possibly carrying thermophilic bacteria on their fur. Ephydrid flies are also observed feeding on cyanobacterial mats at many of the springs and might also carry cells on their bodies (Brock 1994; National Park Service 2004). While Park regulations have not allowed for a test of the animal transport hypothesis, animal vectors would not be a sufficient mechanism for the global distribution of thermophilic bacteria.

Airborne dispersal remains the most viable mechanism to explain the global distribution of closely related thermophiles. Two questions must be answered to support the hypothesis: (i) how might thermophilic bacteria become aerosolized, and (ii) how long can airborne thermophiles maintain viability. This study addresses the first point and demonstrates that updrafts of steam from hot springs may transport thermophilic bacteria into the air, albeit in low numbers. The fact that any bacterial gene sequences at all were detected in the steam, especially one sequence directly linked to the point source of the steam (Spring AT-1) strongly suggests that steam transport occurs. Additionally, other sequences were found in the steam and these will be used in future studies to search for identical sequences in the spring. The similarity (>97%) shared by some clones with thermophilic sequences, while encouraging, cannot be taken as proof that these steam sequences are affiliated with thermophilic bacteria, either from Spring AT-1 or another source. As the sequence database grows for Spring AT-1 and other thermal systems, it will become easier to make comparisons between different communities and with sequences isolated from possible transport vectors (e.g., steam, animal, dust).

4.4 Sequence Divergence In Yellowstone

The 3% sequence variance found between a Firmicutes-like sequence from Spring AT-1 and a cloned sequence from Octopus Spring (Lower Geyser Basin, YNP; **Table 1**), may be too large to have resulted from evolution and divergence within the park. However, 0.4 to 2.2 million years may be sufficient time for Aquificales sequences sharing >99% sequence identity to have diverged from a common ancestor (Ochman and Wilson 1987; Moran et

al. 1993; Ochman et al. 1999). The paucity of sequences sharing 100% sequence identity between Spring AT-1 and other YNP springs may reflect differences in spring chemistries; a sufficiently low rate of transport and exchange that allows for the evolutionary isolation of individual communities; and also the limits of the existing sequence databases to make adequate comparisons between hot spring communities. The apparent absence of sterile hot springs in Yellowstone, however, suggests that viable cells are transported at levels high enough to result in the rapid colonization of new hot springs. New hot springs erupt in the Mammoth Hot Springs complex each year, for example, and these are colonized within days or weeks (Bargar 1978; Bonheyo *unpublished*; NPS *personal communications*). Ongoing studies of different hot springs at Mammoth will provide the data necessary to make comparative analyses between isolated microbial communities from hydrothermal environments with nearly identical physical and chemical attributes. It may be possible with geological dating methods to further constrain the age of specific thermal features and their affiliated microbial communities. Further, with a better understanding of bacterial transport mechanisms and rates, it may be possible to determine the time of microbial community establishment and the extent of species exchange among geographically isolated communities.

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