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Phototrophic Phylotypes Dominate Mesothermal Microbial Mats Associated with Hot Springs in Yellowstone National Park

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Abstract The mesothermal outflow zones (50–65°C) of geothermal springs often support an extensive zone of green and orange laminated microbial mats. In order to identify and compare the microbial inhabitants of morphologically similar green–orange mats from chemically and geographically distinct springs, we generated and analyzed small-subunit ribosomal RNA (rRNA) gene amplicons from six mesothermal mats (four previously unexamined) in Yellowstone National Park. Between three and six bacterial phyla dominated each mat. While many sequences bear the highest identity to previously isolated phototrophic genera belonging to the Cyanobacteria, Chloroflexi, and Chlorobi phyla, there is also frequent representation of uncultured, unclassified members of these groups. Some genus-level representatives of these dominant phyla were found in all mats, while others were unique to a single mat. Other groups detected at high frequencies include candidate divisions (such as the OP candidate clades) with no cultured representatives or complete genomes available. In addition, rRNA genes related to the recently isolated and characterized photosynthetic acidobacterium

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"*Candidatus Chloracidobacterium thermophilum*" were detected in most mats. In contrast to microbial mats from well-studied hypersaline environments, the mesothermal mats in this study accrue less biomass and are substantially less diverse, but have a higher proportion of known phototrophic organisms. This study provides sequences appropriate for accurate phylogenetic classification and expands the molecular phylogenetic survey of Yellowstone microbial mats.

Introduction

Geothermal features in Yellowstone National Park (YNP) have a rich history of microbiological investigation. Many studies have focused on extreme microbial life that inhabit spring waters and sediments of high-temperature pools in YNP, sometimes with extremes of pH or other harsh ecological selections [1–5]. Often, these high-temperature geothermal springs cool to form mesothermal outflows (50–65°C), which are commonly colonized by brightly colored microbial mats. The mesothermal mats are the most conspicuous microbiological feature seen by visitors to the park, thriving where macrobiotic life is notably absent. The mats are typically orange or green on their surface, with one or more colored layers underneath, and vary in thickness from a few millimeters to several centimeters. Microbial mats are known to harbor a broad phylogenetic diversity of organisms and historically have been a source for the discovery of novel microorganisms [1, 6–13].

Previous studies have characterized the biogeochemistry of specific Yellowstone springs [2, 4, 6, 11, 14–17]. Investigators have isolated organisms from some of these environments [6, 8, 12, 18–22], and dominant populations have been studied using electrophoretic and sequence analyses [14, 23]. Ribosomal RNA-based gene sequence studies have provided information mainly on the nature of microbial communities associated with thermal springs [4, 24, 25], including evidence for variability in community composition based on temperature and depth profiles [3, 24, 26, 27]. Select sites such as Octopus Spring in the White Creek group have been particularly well characterized [21, 23, 28, 29] by cultivation and molecular approaches, while others, for example Obsidian Pool, have yet to see successful cultivation of representatives of novel phylum-level groups identified by molecular approaches.

To compare the phylogenetic composition of morphologically similar microbial mats, we conducted a culture-independent rRNA gene sequence-based characterization of six microbial mats associated with the mesothermal zones of five geothermal hot springs in YNP. The mats we chose to compare were somewhat distinct from each other and, in general, do not represent extremes of pH, mineral content, or temperature.

Materials and Methods

Six mats from five springs were examined in this study. Spring water temperature and pH were measured at each site, and water samples were collected and stored frozen until analyzed with the PerkinElmer ICP-OES 3000 (Waltham, MA). Analyses for ion and metal content (shown in Table 1) were performed to the manufacturer's specifications. Mat samples were harvested using flame and ethanol-sterilized equipment from physically similar mats, specifically mats with orange, red, and green laminated layers. Stratified mat layers were separated if feasible (Online resource 1; the supplemental table shows the number of layers extracted from each mat and the sequences derived from each layer). The uppermost layer of each mat is designated as "top," while all layers beneath are designated as "deep" for purposes of comparing the phylogeny associated with the uppermost vs. lower layers. Specimens were frozen in a liquid nitrogen dewar for transport to the laboratory, where samples were stored at −80°C until DNA extraction.

Description of Geothermal Springs and Associated Mats

Obsidian Pool is a high-temperature (80°C) sulfate and hydrogen-rich spring with a pH of 6.5. Outflow channels (55°C) create mats characterized by green and orange colors, often with a silica-rich crust that overlies (or intercalates with) the cells of the mat itself.

Octopus Spring is an alkaline siliceous spring that hosts lush green mats with bright orange underlayers in the radiating effluent channels. The mats tend to grow in size and complexity as the water drains away from the main vent and gradually cools. The water overlying the mats on the south side of the spring is approximately pH 7, and the temperature of the water is 62°C. Octopus Spring has received widespread attention from the microbiology-related research community for more than 40 years [1, 3, 7, 21, 23, 27, 29–32].

A small spring is located near Fairy Falls Bridge on the northwest side of the steel bridge that crosses the Firehole River at the Fairy Falls trail head in the Midway Geyser Basin. This spring harbors mats that also exhibit the typical green surface with thick orange underlayers as the water (pH 9, 55.5°C) empties to the Firehole River.

Queen's Laundry, a hot spring similar to Octopus Spring, has a rich, few-centimeters-thick, multi-layered orange mat that covers ~1,500 m^2 of area. Vent water (pH 8, ~88°C) exits the spring to the northwest, where it quickly cools to ~67°C and fans out across the active mesothermal mat zone. Two distinct mat regions are supported by this spring, herein referred to as Queen's Laundry North and Queen's Laundry Cabin (this spring is alternatively known as Red Terrace Spring in some of the YNP literature).

Table 1 Water chemistry attributes, location, and biodiversity statistics of YNP hot springs and associated microbial mats

	Octopus Spring	Queen's Cabin	West Thumb	Queen's North	Fairy Falls	Obsidian Pool
pH	7	8	7	8	9	6.5
Temp (°C)	50	67	62	67	60	55
YNP Region	Lower Geyser Basin	Lower Geyser Basin	Lake Region	Lower Geyser Basin	Midway Geyser Basin	Mud Volcano
Thermal inventory #	LWCG138	LSMG014	Not available	LSMG014	MRCG043	MV007
Latitude	44°32′2.47″	44°33′49.02″	44°25′13.69″	44°33′49.02″	44°30′58.69′	44°36′35.91″
Longitude	110°47′52.37″	110°52′14.17″	110°34′21.28″	110°52′14.17″	110°49′58.78″	110°26′18.61″
Sequences[b]	207	124	565	81	519	285
S_{obs}/S_{chao1}	19/45.8	18/18.4	28/35.5	14/15	90/135	41/54.1
Singletons	8	3	6	3	36	15
Good's %[c]	96.2	97.6	98.9	96.2	93.1	94.7
Analyte (detection limit, mg/L)[a]						
As (0.0320)	1.48	1.04	1.03	1.04	1.54	BDL
B (0.0080)	2.64	3.20	1.90	3.20	2.76	0.89
Ca (0.0100)	0.56	0.65	0.51	0.65	0.45	**27.36**
K (0.0940)	13.85	12.27	10.36	12.27	10.87	**22.15**
Li (0.0021)	3.25	0.48	1.84	0.48	3.19	0.31
Mg (0.0003)	BDL	0.04	0.04	0.04	BDL	**12.84**
Na (0.0070)	**277.43**	**303.03**	**243.42**	**303.03**	**320.46**	66.83
S (0.1100)	5.59	7.01	8.17	7.01	4.94	**12.28**
Si (0.0040)	**130.68**	**142.69**	**115.80**	**142.69**	**146.03**	95.10

[a] Bold analyte values highlight differences in chemistry between Obsidian Pool and the other springs

[b] Number of bacterial sequences used for alpha diversity calculations in this table

[c] Good's estimate of sampling coverage=(1−singletons/total sequences)

A small, boiling spring near the West Thumb geyser basin has an outflow channel that is 50 m long and rich with centimeters-thick green and orange mats. Water exits the spring at 92°C and pH 7, rapidly cooling down in the runoff channel to 62°C where the mats are firmly established. These mats also accumulate silica and build up in laminated layers that accrue as a prominent rock formation on the west side of Yellowstone Lake. Herein, we refer to this unnamed spring as the "West Thumb Spring."

Nucleic Acid Extraction and rRNA Gene Amplification

Genomic DNA was extracted from six mats using mechanical disruption ("beadbeating") and solvent extraction [33]. Four of the mats contained discrete colored layers, and were separated into two or more layers such that each colored band (1–3 mm) was processed individually (see Online resource 1). Two of the mats were not cleanly stratified and were therefore processed as a single layer. "Universal" primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 1391R (5′-GACGGGCGGTGWGTRCA -3′) [34] were used for PCR amplification of small-subunit ribosomal RNA (SSU rRNA) genes.

Sequence and Data Processing

PCR products were cloned, and sequences were generated on a MegaBACE 1000 (GE) as previously reported [4]. DNA sequences (average length, ~750 nucleotides) were processed with the Xplorseq open source software package [35], aligned with the SINA aligner provided on the SILVA website [36] and added to the guide tree provided with the SILVA database (version 104 SSU Ref) by parsimony insertion with the ARB software package [37]. Phylogenetic identities were determined for each sequence by exporting the SILVA 104 taxonomy lines from ARB. Each taxonomic classification was used as a "bin," or Operational Taxonomic Unit (OTU), in order to compute biodiversity statistics. For each mat, values calculated for observed species richness (S_{observed}, [38]), predicted species richness, (S_{Chao1}, [38]), and Good's coverage [39] are reported in Table 1. In order to compare the similarity of the groups of organisms found in each mat, we employed the Morisita–Horn index [40] to calculate a pairwise similarity score for all mats (Morisita–Horn statistics and graphic plot generated by "Explicet," unpublished software, Charles E. Robertson).

Phylogenetic Tree-Based Relationships to Sequences Deposited in the SILVA Database

Initial phylogenetic relationships between acidobacterial sequences were established via parsimony insertion (masked with the pos_var_Bact104 filter) into the guide tree included with the SILVA 104 database. The parsimony-derived taxonomies were validated by casting 1,000 bootstrap trees with RAxML (version 7.2.3) [41]. The bootstrap scores were annotated on the best scoring maximum likelihood tree found by RAxML's ML search function. A total of 1,812 sequences (average length, ~750 nucleotides) were deposited in GenBank with accession numbers FJ884901-FJ886712.

Results and Discussion

In this study, we generated long (~750 nucleotides on average) rRNA sequences with Sanger sequence technology in order to identify organisms at a higher level of phylogenetic resolution than afforded by high-volume datasets with shorter sequences. Despite the trend of generating large numbers of short sequences to describe microbial communities, recent work has shown that valid community comparisons are retained when using a limited set of sequences to describe microbial communities [42].

Phylogenetic identifications were assigned to sequences based on parsimony insertion into the SILVA (version 104 Ref) database. We used these taxonomic classifications to bin the sequences into OTUs and measure the microbiological "species" richness for each mat (S_{observed}). Biodiversity statistics (S_{chao1}, Good's coverage) were calculated and indicated reasonable sampling coverage of the most abundant microbial populations in the mats. The statistics also indicated that one of the mats (Fairy Falls) is substantially more diverse than the other mats. Fairy Falls mat was sampled to a depth of over 500 sequences and has threefold higher observed taxa compared to the West Thumb mat (also with >500 sequences). However, although the predicted value of taxa is 50% higher than those actually observed in Fairy Falls, there were relatively few singletons (sequences observed only once) in the sequence collection. Therefore, the sampling coverage (Good's estimate) was still greater than 90% for this most diverse mat.

All mats contained sequences indicative of the phyla Cyanobacteria, Chloroflexi, and Chlorobi, while particular mats also contained a high frequency of sequences representative of Bacteroidetes, Acidobacteria, and the OP (OP9, OP10, OD1) candidate clades (Fig. 1). At the genus level, some members of the mat consortia were shared among all mats (e.g., *Roseiflexus* spp., *Synechococcus* spp., *Candidatus Chloracidobacterium* in five of six mats), while others were only abundant in a single mat (e.g., *Fischerella* spp. in Obsidian Pool and candidate division OD1 in West Thumb Pool). Few archaeal (~4.4% of total clones from the West Thumb mat) and no eucaryal sequences were amplified from mat DNAs with universal primers, and attempts with domain-specific eucaryal and archaeal primers did not yield PCR products.

Taxon assignment (Silva v104)		% Abundance per Mat	Octopus	Queen's Cabin	West Thumb	Queen's North	Fairy Falls	Obsidian
Cyanobacteria	*Synechococcus spp.*		22.0	17.7	35.8	20.7	3.1	0.7
	Fischerella spp.							16.5
	Phormidium spp.		0.5	20.2			1.0	
	Leptolyngbya spp.				0.2		4.8	0.4
	"uncultured"			7.3			11.6	0.7
Chloroflexi	*Roseiflexus spp.*		15.8	1.6	11.5	18.3	11.0	17.5
	Chloroflexus spp.				1.8	6.1	0.2	5.3
	"uncultured"		5.3	8.9	6.4	8.5	5.4	7.0
Chlorobi	"OPB56"		2.4	1.6	1.1	3.7	0.6	5.3
	"BSV26"				3.5		1.5	
	"uncultured"		5.3	2.4	3.2		4.4	
Other Phyla	*Chloracidobacterium spp.*		8.6	4.8	0.5		8.5	12.3
	"uncultured" *Saprospiraceae*		22.0	21.0			1.3	
	OP10		7.2	1.6	12.6	24.4	3.3	4.6
	OD1				11.3			1.1
	Other Bacteria (not listed)		11.0	12.9	12.1	18.3	43.4	28.8

Figure 1 Distribution of bacterial phylotypes across six YNP mesothermal mats. Taxonomies were assigned by parsimony insertion into the SILVA (version 104 SSU Ref) database. Clones generated from multiple layers of a given mat were pooled, and abundance values for each OTU were normalized relative to the total number of sequences generated from each mat (includes only bacterial phylotypes represented by >1% abundance of total sequences). *Lightest gray shading* indicates abundance greater than 5% and less than 15%, *dark gray shading* indicates abundance greater than 15% and less than 20%, *black shading* indicates abundance greater than 20%

Synechococcus spp. were the dominant Cyanobacteria in three of the six mats analyzed (Fig. 1). In Octopus Spring, sequences indicative of *Synechococcus* spp. were the only abundant cyanobacterial phylotype and bear the highest identity (<5% sequence difference, data not shown) to sequences recovered in previous studies of this spring [23, 32, 43]. In contrast, *Fischerella* spp. rRNA genes dominated the Obsidian Pool mat library, and *Synechococcus* spp. represented <1% of sequences. *Leptolyngbya* spp. were only conspicuous in the Fairy Falls mat library. *Roseiflexus* spp. (phylum Chloroflexi) rRNA genes were conspicuous in all mat libraries, particularly in the deeper layers of the mats (Fig. 2, ~28% vs. 12%). This pattern contrasts with that of the Cyanobacteria, which are at least twice as prevalent in top layers (see also Fig. 2). Similar patterns of stratification by Cyanobacteria and Chloroflexi groups have been noted in previous Yellowstone mat studies [2, 44].

Sequences representing the phylum Chlorobi ("green sulfur bacteria"), another predominantly phototrophic phylum, comprised between ~4% and ~8% of the sequences

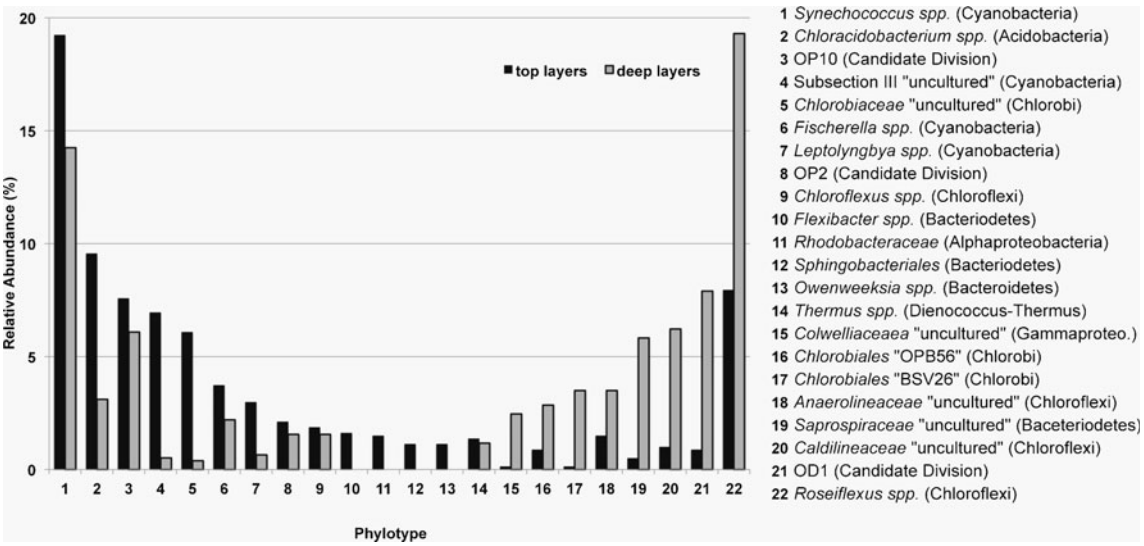

Figure 2 Opposing rank abundance histograms (top layers vs. deep layers) of the 22 most prevalent bacterial phylotypes in all uppermost layers of mats compared with all deeper layers of mats (includes only bacterial phylotypes represented by >1% abundance of total sequences)

generated from each mat. Sequences representing an uncultured, unnamed member of this phylum were more common in the top layers of the mats (Fig. 2, phylotype 5; see also Online resource 1). Conversely, sequences indicative of the Chlorobi clades "OPB56" and "BSV26" were predominant in the deeper layers (Fig. 2, phylotypes 16 and 17; see also Online resource 1).

Sequences indicative of the phylum Acidobacteria accounted for ~7% of the total sequences determined (the fourth most abundant phylum detected) and were threefold more prevalent in the top layers of mats than in deeper layers (Fig. 2, phylotype 2). Nearly all of these sequences bear high identity (>95%) to the rRNA of the recently isolated photoheterotrophic acidobacterium "*Candidatus Chloracidobacterium thermophilum*" (GenBank accession no. EF531339.1, Fig. 3). Sequences representative of this phylotype were detected in all mats but one, and three mats had particularly high representation of *Chloracidobacterium* spp. rRNA genes: Octopus Spring (8.6%), Fairy Falls Bridge (8.5%), and Obsidian Pool (12.3%). This suggests abundant and widespread distribution of organisms similar to the *C. thermophilum* cultivar in the YNP mats we studied.

Sequences related to rRNA genes of proposed "candidate" bacterial phyla were identified in all mat libraries, sometimes abundantly, but not necessarily uniformly (Fig. 1). Sequences indicative of the OD1 candidate phylum, for instance, comprised 11.3% of the West Thumb mat library but were not conspicuous in the other mats. Ribosomal RNA genes representative of candidate phylum OP10, however, were detected in all mats and were particularly abundant in the Queen's Laundry North mat. Here, they were even more abundant (24.4%) than *Synechococcus* spp. (20.7%) or *Roseiflexus* spp. (18.3%).

Sequences most closely related to those of the *Saprospiraceae* family (phylum Bacteroidetes) were particularly abundant in the Octopus Spring (22%) and Queen's Cabin (21%) mats, but were not conspicuous in the other mats. Members of the *Saprospiraceae* have been reported to inhabit pelagic zones of freshwater habitats and are abundant in activated sludge wastewater treatment systems [45]. To our knowledge, they have not been previously reported in microbial mat environments.

Archaeal sequences were not abundant in these mats. One sequence that represents the terrestrial hot spring crenarchaeote group was recovered from Queen's Laundry mat, and one representative of the deep-sea hydrothermal vent euryarchaeote group 6 was recovered from the Octopus Spring mat. The only substantial prevalence of archaeal sequences in any of the mats were 26 sequences of the crenarchaeote group "*Candidatus Nitrosocaldus yellowstonii*" from a deep layer of the West Thumb mat (~20% of the clones generated from that layer), suggesting active archaeal ammonia oxidation as a possible biochemical theme in this system [46].

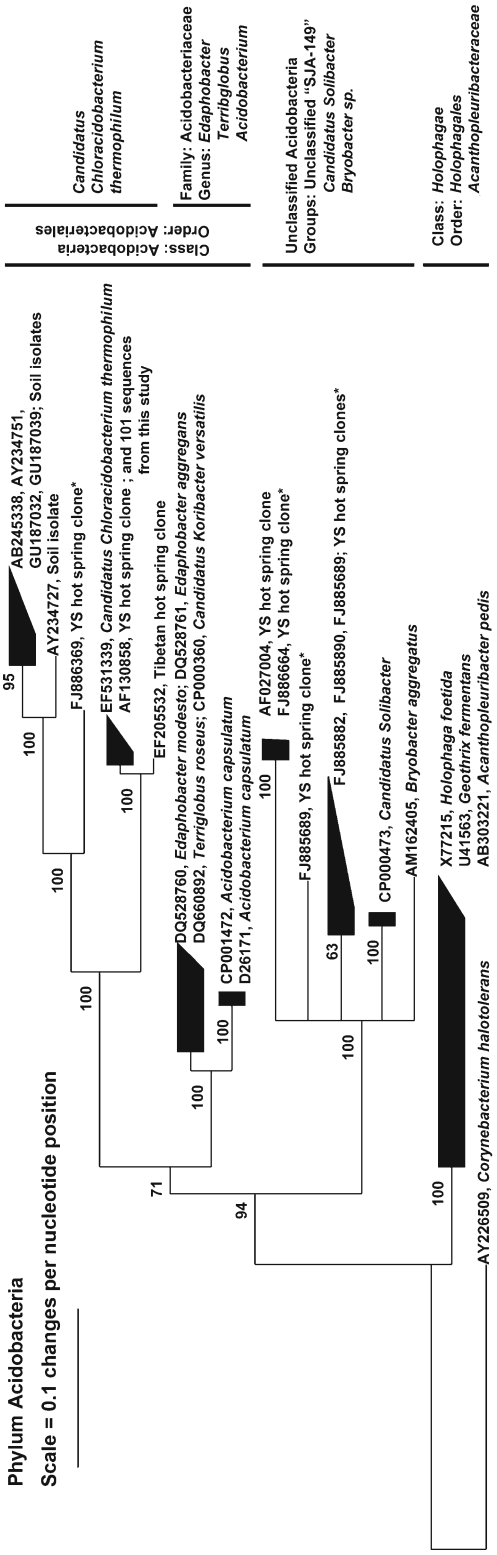

Figure 3 Schematic representation of a phylogenetic dendrogram of acidobacterial sequences (nodes with less than 50% bootstrap support were collapsed). The rRNA sequence of *Corynebacterium halotolerans* (AY226509) was used to root the tree

In order to compare the phylogenetic composition of the mats we studied to each other, we employed the Morisita–

Horn (MH) index [40] to elucidate similarity between the mat communities based on the clone library data (Online resource 2). We observed that the two mat communities that bear the highest similarity to each other are West Thumb and Queen's North (MH score ~0.8). These mats have similar temperature, pH, and ion content. When all mat communities are compared to the Octopus Spring mat community (based on clone library data), we find that both of the Queen's Laundry mats and West Thumb mat have MH scores >0.65, which indicates some similarity among these four sites. Fairy Falls mat and Obsidian Pool mat communities are more distantly related to Octopus (MH scores of approximately 0.4). Obsidian Pool mat is the furthest outlier, with MH scores of <0.5 when compared to all other mats. The chemistry of Obsidian Pool is also the most different compared to the chemistry of all four other springs (see Analyte concentrations in bold type, Table 1).

We investigated whether the variation observed in phylogenetic composition between mat communities correlated to measured geochemistry. Linear regressions were used to correlate observed sequence relative abundances with ion content from the pools (for example, Chlorobi sequence abundance versus sulfur concentration). These regressions resulted in values less than 0.8 for all OTU/ion pairs tested and thus were deemed unconvincing (data not shown). A lack of correlation between geochemistry and phylogeny was also reported by Papke and colleagues, in a study that investigated different springs worldwide and found differences in phylogenetic composition were not explained by the chemical attributes that were measured [25].

In conclusion, we found general similarities at the phylum level in rRNA gene sequence libraries generated from different green–orange mesothermal microbial mats in Yellowstone National Park. We report the frequent prevalence of a recently isolated acidobacterial phylotype, and we note conspicuous members of the Cyanobacteria, Chloroflexi, and Chlorobi that have no close relatives that have been isolated or otherwise classified. We also identify at least one phylotype not previously reported to be associated with Yellowstone mats (uncultured members of the *Saprospiraceae* in two of six mats). Moreover, our analyses indicate relatively low diversity overall in these thinly stratified microbial mats, which contrasts with the extravagant diversity reported in thick, intensely stratified, temperate hypersaline mat systems [47–50]. We speculate that the difference in diversity content of the two types of mat systems is due to their relative thickness, stabilities, and geochemical opportunities. Hypersaline mats studied by rRNA sequences, such as the Guerrero Negro mat system, are typically well developed (>5-cm thickness) and stable over years, and lie protected under a meter of seawater [47, 48]. In contrast, Yellowstone mesothermal mats typically are thin (<1 cm), covered by only a few centimeters of water, are sometimes disturbed by changes in water flow, weather, and wildlife, and are subject to seasonal environmental variations in UV intensity and air temperature. The more stable and massive hypersaline mats are expected to accrue more biochemical opportunities than the less-developed Yellowstone mats and thereby expand the diversity supported by the local ecosystem. Relatively low diversity is also encountered in thin-bedded marine mats, consistent with the notion that accumulation of biomass results in increased diversity [51, 52]. Although the YNP mats tend to be thin, we observed a different distribution of phylotypes in top layers compared with the deeper layers of mats, which indicates mat stratification and possible variation in community function at different depths in the mats. This study expands the knowledge of Yellowstone mat ecosystems into members that may not be morphologically conspicuous but because of their abundance must be major contributors to the local ecosystem.

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