Applied and Environmental Microbiology, Apr. 2009, p. 2464–2475 Vol. 75, No. 8
0099-2240/09/$08.00+0 doi:10.1128/AEM.01802-08

Formation of Multilayered Photosynthetic Biofilms in an Alkaline Thermal Spring in Yellowstone National Park, Wyoming[▽]

Sarah M. Boomer,[1]* Katherine L. Noll,[2] Gill G. Geesey,[3] and Bryan E. Dutton[1]

Western Oregon University, Department of Biology, Monmouth, Oregon 97361[1]; Quality Schools International, Chengdu, China 610000[2]; and Montana State University, Department of Microbiology, Bozeman, Montana 59717[3]

Received 4 August 2008/Accepted 9 February 2009

In this study, glass rods suspended at the air-water interface in the runoff channel of Fairy Geyser, Yellowstone National Park, WY, were used as a substratum to promote the development of biofilms that resembled multilayered mat communities in the splash zone at the geyser's source. This approach enabled the establishment of the temporal relationship between the appearance of *Cyanobacteria*, which ultimately formed the outer green layer, and the development of a red underlayer containing *Roseiflexus*-like *Chloroflexi*. This is the first study to define time-dependent successional events involved in the development of differently colored layers within microbial mats associated with many thermal features in Yellowstone National Park. Initial (1-month) biofilms were localized below the air-water interface (60 to 70°C), and the majority of retrieved bacterial sequence types were similar to *Synechococcus* and *Thermus* isolates. Biofilms then shifted, becoming established at and above the air-water interface after 3 months. During winter sampling (6 to 8 months), distinct reddish orange microcolonies were observed, consistent with the appearance of *Roseiflexus*-like sequences and bacteriochlorophyll *a* pigment signatures. Additionally, populations of *Cyanobacteria* diversified to include both unicellular and filamentous cell and sequence types. Distinct green and red layers were observed at 13 months. *Planctomycetes*-like sequences were also retrieved in high abundance from final biofilm layers and winter samples. Finally, biomass associated with geyser vent water contained *Roseiflexus*-like sequence types, in addition to other high-abundance sequence types retrieved from biofilm samples, supporting the idea that geothermal water serves as an inoculum for these habitats.

Biofilms are widely recognized as the way that most microbes exist in natural habitats (10–12), often developing into thick mats on various substrata associated with geysers and thermal springs in places such as Yellowstone National Park (53). In the first published microbiological studies of near-boiling pools in Yellowstone's Lower Geyser Basin, an immersed glass slide approach was used to recover biofilm-forming microbial communities containing thermophilic *Cyanobacteria*, pink and yellow filaments, and many colorless rods (7, 8). Dispersal of microorganisms from biofilms has been investigated using 16S rRNA studies of geothermal vent source water and downstream travertine deposit samples at Angel Terrace, a carbonate spring in Mammoth Basin, Yellowstone National Park (18, 19). Using an artificial substrate and in situ kinetic apparatus at Angel Terrace, Kandianis et al. recently demonstrated that biomass serves as a catalyst in the precipitation of calcium carbonate (27). Regrowth of cyanobacterial mat communities after excision and removal at the alkaline Octopus Spring in the Lower Geyser Basin was investigated by Ferris et al. (16). In an effort to address how biofilms influence sinter formation, Cady and Farmer (9) observed that the presence of hyperthermophilic biofilms influenced the development of spicular geyserite by providing a preferred substratum for opaline silica precipitation. In a follow-up study, Blank et al. observed that communities in the subaqueous environment of alkaline, silica-depositing springs were similar to those associated with spicular geyserite at the air-water interface within the same spring (3). However, none of these studies has examined biofilm growth using artificial substrata to systematically evaluate community succession over time.

Fairy Geyser represents a constantly erupting alkaline geothermal feature. Its vent waters (70 to 90°C) almost constantly erupt and splash, supporting the growth of extensive multilayered mat communities (35 to 60°C and pH 7.5 to 8.5) that form just above the water, on top of the sinter which surrounds the main geyser vent (5, 6). Fairy Geyser splash mats are composed of a green surface layer of *Cyanobacteria* and a red underlayer dominated by *Roseiflexus*-like *Chloroflexi* (5, 6). In monitoring Fairy Geyser since 1998, we have regularly observed the formation of similar multilayered photosynthetic microbial mats in splash zones at the cooler air-water interface (35 to 40°C) along the runoff channel or on debris that has fallen into the runoff channel (S. Boomer, unpublished results). Consequently, we hypothesized that solid substrata suspended in the Fairy Geyser runoff channel should support biofilm development and serve as a simple means for monitoring successional events over time, including the development of multilayered communities. Because *Roseiflexus*-like *Chloroflexi* from comparable red-layer communities in Yellowstone have been shown to be nonsulfur photoheterotrophs that metabolize under low-intensity light (6), we further hypothesized that red underlayer formation would require the presence of a shielding green layer and would thus form after initial colonization by *Cyanobacteria*. In addition to decreasing the light intensity, the photoautotrophic *Cyanobacteria* would provide organic compounds for the photoheterotrophic *Chloroflexi*, which typically consume

* Corresponding author. Mailing address: Department of Biology, Western Oregon University, 345 Monmouth Ave., Monmouth, OR 97361. Phone: (503) 838-8209. Fax: (503) 838-8072. E-mail: boomers@wou.edu.

▽ Published ahead of print on 13 February 2009.

cyanobacterial photosynthate in nature (40). Finally, we hypothesized that *Roseiflexus*-like *Chloroflexi* would be present in geothermal water at the vent source, providing an inoculum for substrata in the runoff channel.

To test these hypotheses, we characterized the microbial community that accumulated at the air-water interface of sterile glass rods suspended in the thermal runoff at Fairy Geyser. Owing to environmental resource impact and winter access issues, this report encompasses two separate year-long studies. The first study (2004-2005) investigated the succession of the rod-associated communities during the first 3 months of colonization in the summer season, while the second study (2006-2007) investigated the succession after longer periods (6 to 8 months) of colonization during the winter season. In both cases, some replicates were maintained for 13 months, producing thick and gelatinous mat-like biofilms with outer green and inner red layering. For samples from all time points, we assessed biofilm accumulation by using microscopy, pigment analysis, and 16S rRNA studies, targeting both general bacteria and *Chloroflexi*. Although aforementioned Yellowstone biofilm studies have reported growing monolayers on artificial substrates or performed disturbance and recovery studies of existing mat systems in Yellowstone, this study represents the first report of multilayered photosynthetic mats being generated and studied in this manner.

MATERIALS AND METHODS

Biofilm formation field methods. Our study site was at Fairy Geyser, Yellowstone National Park, WY (44°32′53″N, 110°51′68″W), a National Science Foundation (NSF) Microbial Observatory site. Previously, only the splash mats at the air-water interface (48 to 53°C, pH 8.0) around the vent source (Fig. 1A, panel *a*) were described (5, 6). In accordance with low-impact requirements defined by the Yellowstone Center for Resources (Christie L. Hendrix, Research Permit Facilitator, personal communication), we selected a location 100 m downstream from the vent source that provided adequate space for placing our artificial substrata for colonization. Specifically, we deployed sterile glass rods (20 cm long, 1 cm in diameter), suspending them partially in the geothermal runoff by using a sterilized clothespin that was secured onto a dead branch placed across the runoff channel (Fig. 1A, panels *b* and *c*). At the times of placement, 3 to 5 cm of each rod was submerged in the runoff, leaving a 5- to 7-cm length between the waterline and the point of attachment. We chose glass rods over slides because a more uniform distribution of biomass was achieved with the former. Two 13-month studies were conducted between 2004 and 2007. For the first study, 12 rods were deployed on 30 May 2004; 4 rods were subsequently collected on 30 June 2004 (1 month), 14 September 2004 (3 months), and 8 July 2005 (13 months). For the second study, 12 rods were deployed on 9 June 2006; 4 rods were subsequently collected on 20 December 2006 (6 months), 24 Feb. 2007 (8 months), and 18 July 2007 (13 months). For all 1- to 8-month-incubation samples, the portion of each rod containing visible biofilm was cut with a glass cutter and transferred aseptically on-site to a sterile 50-ml conical plastic screw-cap test tube, frozen at −20°C within 2 h of collection, shipped on dry ice to the laboratory, and stored at −80°C. For all 13-month incubation samples, the biomass associated with each rod was separated with sterile forceps and scalpels into outer green and inner red layers while in the field. Separated layers were transferred to separate 1.5-ml microcentrifuge tubes, frozen at −20°C within 2 h of collection, shipped on dry ice to the laboratory, and stored at −80°C. Temperature and pH within the Fairy Geyser splash mat were measured on 9 June 2006 and 18 July 2007, and those within 13-month biofilms were measured on 18 July 2007 as described previously (5).

Water sampling and aqueous-phase chemistry. To assess biomass suspended in the water emerging from the vent source, water samples were collected on 9 June 2006 using sterile, nitric acid-cleaned 2-liter Nalgene polypropylene Boston Round bottles. Within 2 h of collection, six 1-liter volumes were filtered through sterile Millipore Isopore polycarbonate membranes (37-mm diameter, 0.4-μm pore size) and the membranes placed in a 50-mm-diameter tight-lid petri dish (FisherBrand, Pittsburgh, PA), frozen at −20°C, shipped on dry ice to the laboratory, and stored at −80°C. To assess aqueous-phase chemistry at the source of the geyser and at the biofilm experimental site, water samples were collected from these locations on 9 June 2006 and 18 July 2007 using sterile, nitric acid-cleaned 2-liter Nalgene polypropylene Boston Round bottles. Nitrate was determined by the Hach cadmium reduction assay using the AccuVac NitraVer 5 reagent, sulfate by the Hach Sulfa Ver 4 method using Sulfa Ver 4 reagent, and sulfide by the methylene blue method using the Hach sulfide reagents (Hach water analysis handbook, 3rd ed., 1997; Loveland, CO). A Hach DR/890 portable colorimeter was used to measure color development in the above-mentioned chemical assays. Water temperature and pH at both of these locations were measured on 9 June 2006 and 18 July 2007 as described previously (5).

Biofilm sample preparation and biomass determination. To compare similar amounts of biofilm growth from each time point, efforts were made to measure, resuspend, and aliquot defined amounts of each sample. For 1- to 8-month samples, 10 ml of glucose-Tris-EDTA buffer (pH 7.5) was added to each tube, followed by 5 to 10 min of vortex agitation and scraping using a sterile scalpel. Biofilm resuspensions were homogenized and subdivided into 1-ml aliquots and centrifuged at 10,000 × *g* for 4 min at room temperature to pellet cells, and wet weight was determined following removal of the buffer supernatant. After 13-month green- and red-layer samples previously dissected from the rods in the field were weighed, each layer was separated into 0.1-g aliquots with sterile forceps and scalpels.

Microscopy. All microscopy was performed using an Olympus BX41 microscope equipped with a BX-URA2 fluorescence illuminator and a DP71 digital camera system (Olympus America, Inc., Melville, NY). Biofilm sample aliquots were examined under phase contrast light and fluorescence by using an Olympus U-MWIB2 filter set (with excitation at 460 to 490 nm) to assess for red chlorophyll *a* (Chl *a*) autofluorescence. Biomass was removed from water filter samples by combining each filter with 6 ml glucose-Tris-EDTA in a 15-ml conical tube and gently rocking the tube overnight at 4°C. Buffer containing removed cells was concentrated as described above for biofilm samples.

Pigment analysis. Total pigment was extracted from rod biofilm samples by using 100% methanol as previously described (5). Biomass associated with each biofilm homogenate (1- to 8-month samples) and each biofilm layer (13-month green and red layers) was distributed into 0.02-g subsamples, and the absorbance of the methanol extract from 200 to 1,000 nm was measured to determine the presence of Chl *a* or bacteriochlorophyll *c* (Bchl *c*) (664 to 669 nm) and Bchl *a* (768 to 771 nm). Pigments were quantified as previously described (43) and reported in μg/g wet mat weight. There was insufficient biomass from the water filter samples for an extraction to be performed, as pigment content was below detection limits.

DNA extraction, PCR amplification, and cloning. Genomic DNA was isolated from whole water sample filters, 0.02 g of each biofilm homogenate (for 1- to 8-month samples), and 0.02 g of each biofilm layer (for 13-month green and red layers), as previously described (5), by using a Mini-Beadbeater (Biospec Products, Inc., Bartlesville, OK) at a setting of six for 60 s, and this process was repeated two times. Two different primer sets were used to amplify portions of 16S rRNA genes: bacterial primers (8FPL and 1492 RPL) (45) and *Chloroflexi*-specific primers (CCR-344-F and CCR-1338-R) (35). All PCR amplification procedures and cloning steps were performed as previously described (5). Forty-six to 59 bacterial clones generated with bacterial primers and 23 to 34 *Chloroflexi* clones from each sample were subsequently sequenced.

Nucleotide sequence analyses. Nucleotide sequence analysis was performed at Oregon State University's Center for Genome Research and Biocomputing, using an ABI Prism 3730 genetic analyzer with ABI Prism and DNA Sequencing Analysis Software (version 5.2). One end read was determined for each clone, with obtained sequences averaging 870 bp in length. Each data set was analyzed using Bellerophone (25), with predicted chimeric sequences culled. In October 2008, the sequences of all final clones were compared with those in the GenBank database by using the Basic Local Alignment Search Tool (BLAST) network service (2) and with those in the Ribosomal Database Project (RDP) database by using the RDP classifier (52).

Statistical analysis. Our screening process was tested by statistical analyses to evaluate whether total diversity was covered by screening 355 bacterial clones (the total number amplified and analyzed using bacterial primers). Two types of analyses were used. Coverage values were calculated as described in references 44 and 55. In addition, rarefaction analysis was performed to determine the number of unique operational taxonomic units (OTUs; phyla) as a proportion of the estimated total diversity. Calculations were performed using Analytic Rarefaction version 1.3 software (http://www.uga/~strata/software/Software.html).

Phylogenetic analyses. *Chloroflexi* library-derived sequences were analyzed with known *Chloroflexi* representatives from the NCBI taxonomy database. Two

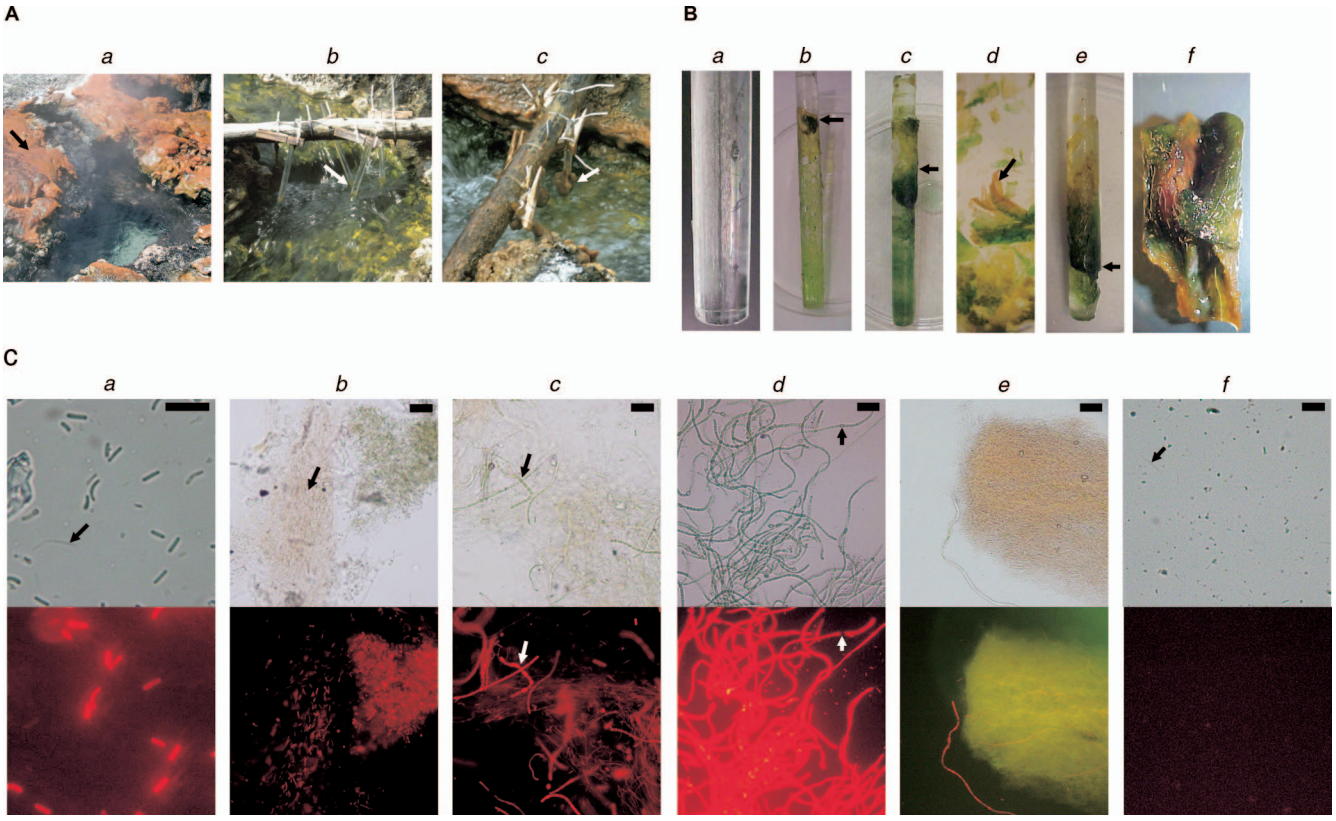

FIG. 1. Color plates showing macroscopic site and microscopic biofilm images. (A) Site images. Image *a* shows the natural splash mats located at the air-water interface by Fairy Geyser; the arrow indicates the community that has previously been sampled and described. Image *b* shows glass rods suspended in the Fairy Geyser runoff after 1 month (June 2004); the arrow indicates the air-water interface. Image *c* shows glass rods suspended in the Fairy Geyser runoff after 13 months (July 2007); the arrow indicates the biofilm samples growing at and above the air-water interface. (B) Macroscopic images of biofilm samples. Images *a*, *b*, *c*, and *e* show whole glass rod biofilm samples from 1 month (June 2004), 3 months (September 2004), 6 months (December 2006), and 8 months (February 2007), respectively. For images *b*, *c*, and *e*, arrows indicate the location of air-water interface at the time of collection. Image *d* shows the 13-month biofilm being homogenized during sample preparation; the arrow indicates notable reddish orange sheets that began to appear at this time point. Image *f* shows a biofilm sample at 6 months that has been removed from the glass rod to reveal a cross-section of the outer green and inner red layers; this entire sample grew at and above the air-water interface. (C) Microscopic images of biofilm and water samples. All top row images show samples viewed under transmitted light; all bottom row images show samples viewed under fluorescence using an Olympus U-MWIB2 filter set (excitation at 460 to 490 nm) to assess for red autofluorescence, indicative of Chl *a*; all bars measure 10 μm. Column *a* shows images from 3-month (September 2004) biofilm samples, which also represented the 1-month biofilm samples; the arrow indicates a nonfluorescing filament. Column *b* shows images from 6-month (December 2006) biofilm samples; the arrow indicates one of several notable reddish orange microcolonies, the filaments of which did not exhibit autofluorescence. Column *c* shows images from 8-month (February 2007) biofilm samples; the arrow indicates one of many filamentous cyanobacterial cell types that began to appear after 6 months. Column *d* shows images from 13-month green-layer samples; the arrow indicates a heterocyst along one of many filamentous cyanobacterial cell types in these samples. Column *e* shows images from 13-month red-layer samples, dominated by reddish orange filament masses that did not exhibit autofluorescence. Column *f* shows images from filtered water biomass; the arrow indicates a nonfluorescing filament.

levels of phylogenetic analyses were performed: first, trees were generated from all *Chloroflexi* library-derived sequences from each sampling time point and used to select representatives from each library (data not shown); then, trees were generated using representatives from across this study. In all analyses, datasets were compiled using the San Diego Supercomputer Center's Biology Workbench (version 3.2; http://workbench.sdsc.edu/) (47) and aligned using CLUSTAL W version 1.81 (49) with identity matrix settings that assigned equal weight to all nucleotides. All phylogenetic trees were generated by parsimony methods using PAUP 4.0b10 (48). All trees were tested for robustness with bootstrap resampling methods. The final alignment was trimmed to correspond with the shorter lengths of our *Chloroflexi*-like sequence (corresponding to bases 243 to 1249 of *Escherichia coli*). *Thermotoga hypogea* (U89768) (15) was used as the outgroup in the final analyses.

Website and database. Long-term monitoring data for Fairy Geyser can also be found at the Red Layer Microbial Observatory Database (http://www.wou.edu/rlmodb) (4).

Nucleotide sequence accession numbers. Sequence data have been submitted to the GenBank database under accession numbers FJ206237 to FJ206805.

RESULTS

Biofilm modeling experimental site. To assess conditions at the natural splash mats by the Fairy Geyser vent source and those at our experimental site, we measured temperature and pH as well as aqueous-phase chemistry in the water and within the biofilm communities. When we compared water conditions at the vent source and biofilm modeling site, we observed predictable gradients involving temperature and pH (Table 1). When we compared biofilm conditions at the air-water interface, we observed that temperature and pH measurements within 13 months of biofilm growth on the glass rod substrates were most similar to those at the natural splash mats at the vent source (Table 1). In terms of assessed aqueous-phase chemistry, sulfide, sulfate, and nitrate levels in the runoff water

TABLE 1. Site data for Fairy vent sources versus those for biofilm experimental sites

Location and parameter	Value for indicated mo and yr	
	June 2006	July 2007
Fairy vent source		
Mat pH	9.5	8.5
Mat temp (°C)	43.5	41.3
Water pH	8.0	7.6
Water temp (°C)	90	81.8
Sulfide level (mg/liter)	0.04	0.02
Sulfate level (mg/liter)	18.00	19.00
Nitrate level (mg/liter)	5.3	1.7
Biofilm experiment site		
Biofilm pH	Too thin to measure	9
Biofilm temp (°C)	Too thin to measure	38.8
Water pH	8.3	8.1
Water temp (°C)	71.1	68.7
Sulfide level (mg/liter)	0.02	0.00
Sulfate level (mg/liter)	13.00	14.00
Nitrate level (mg/liter)	3.20	1.40

dropped between the vent source and the biofilm modeling site (Table 1).

Biofilm characteristics. Over the five time points assessed during these two year-long studies, macroscopic biofilm growth was apparent. Because a portion of the rods had to be suspended in the water to generate a splashing effect that was similar to that at the vent source, we could not avoid having some biofilm growth in the submerged, hotter runoff water. One-month (June 2004) samples (0.04 g) appeared as faint, colorless films on the submerged region of each rod (Fig. 1B, panel *a*). Three-month (September 2004) samples (0.24 g) appeared as thicker (1-mm) green films that covered the submerged region of the rod; at and above the air-water interface, the biofilm appeared more greenish yellow (Fig. 1B, panel *b*). Both 6- and 8-month biofilm samples (0.51 g [December 2006] and 0.56 g [February 2007]) appeared as thin (1-mm) green films that covered the submerged region of the rod; at and above the air-water interface, a thicker biofilm (2 to 3 mm) appeared more greenish yellow, with distinct reddish orange patches visible during biofilm removal (Fig. 1B, panels *c* to *e*). Thirteen-month (July 2007) biofilm samples (6.30 g) consisted of a thin (1-mm) green film that covered the submerged region of the rod and a large gelatinous mass (5 to 15 mm) at and above the air-water interface; the latter appeared externally green (3 to 5 mm), with a distinct red-layer inner core (3 to 5 mm) (Fig. 1B, panel *f*). The 13-month replicate sample from July 2005 was similar (data not shown).

Over the five time points assessed, microscopic evidence of some cell type shifts was also observed (Fig. 1C). In both 1 (June 2004)- and 3 (September 2004)-month biofilm samples, the cell types included unicellular cyanobacterial rods that exhibited red Chl *a* autofluorescence as well as thinner filaments (0.5 to 1 μm in diameter) and unicells that did not autofluoresce (Fig. 1C, panel *a*). In both 6 (December 2006)- and 8 (February 2007)-month biofilm samples, the cell types included unicellular cyanobacterial rods and filaments that exhibited red Chl *a* autofluorescence as well as reddish orange masses of thinner filaments that did not autofluoresce (Fig. 1C, panels *b* and *c*). In terms of 13-month samples (July 2007), outer-green-layer biofilm samples primarily consisted of unicellular cyanobacterial rods and filaments, some with heterocysts, that exhibited red Chl *a* autofluorescence (Fig. 1C, panel *d*); inner-red-layer biofilm samples primarily consisted of reddish orange filament masses that did not autofluoresce (Fig. 1C, panel *e*). The 13-month replicate sample from July 2005 was similar (data not shown). Filtered water biomass contained a variety of unicells and thin filaments (0.5 to 1 μm in diameter), none of which exhibited autofluorescence (Fig. 1C, panel *f*).

Over the five time points assessed, methanol extraction and spectrophotometric absorbance profiles of same-sized biofilm samples were used to characterize and compare the relative levels of green (both Chl *a* and Bchl *c*) and red (Bchl *a*) photopigments (Fig. 2A). Between 1 and 3 months (June to September 2004), biofilm samples showed increasing levels of green pigment. Between 6 and 8 months (December 2006 to February 2007), biofilm samples showed decreasing levels of green pigments as well as Bchl *a* peaks for the first time. In terms of 13-month samples (July 2007), the outer green layer showed levels of green pigments that were similar to levels in the 3-month (September 2004) biofilm; the inner red layer showed less green pigment and the highest levels of red pigment. The 13-month replicate sample from July 2005 was similar (data not shown).

General-bacterial-library analysis and community composition. In this study, we classified 355 16S rRNA clones from biofilm samples over time and filtered water biomass by using BLAST (2) and the RDP classifier (52). The library coverage values, calculated as previously described (44, 55), were between 81 and 89% (Fig. 2B), and calculated rarefaction curves showed a slight tendency to saturation (Fig. 2B), indicating that diversity was not completely sampled. Using BLAST information, we selected 22 clones that represented observed OTU diversity among 12 retrieved phyla (Table 2). We assigned an OTU to a division if it exhibited more than 75% sequence similarity to a cultured representative. We also summarized the retrieval frequencies of the 12 retrieved phyla in each sample library (Table 2 and Fig. 2B). Given our interest in understanding the specific dynamics of *Cyanobacteria* and red *Chloroflexi*, we plotted retrieval for just these groups in Fig. 2A. As shown in Table 2 and Fig. 2B, most retrieved library members from both 1-month (June 2004) and 3-month (September 2004) biofilm samples were *Cyanobacteria*-like. Although 33% *Thermus*-like sequences were retrieved from the 1-month sample, these sequences were retrieved in only low abundances (<10%) after 3 months. For both 6-month (December 2006) and 8-month (February 2007) biofilm samples, most retrieved library members were *Cyanobacteria*-like. Although 28% of the sequences retrieved from the 6-month sample were *Planctomycetes*-like, these sequences were retrieved in only low abundances after 8 months. In terms of 13-month samples (July 2007), most retrieved library members from the outer green layer were *Cyanobacteria*-like, and most retrieved library members from the inner red layer were *Chloroflexi*-like, nearly all *Roseiflexus*-like (Table 2 and Fig. 2A). In both the outer green and the inner red layers, we retrieved many *Planctomycetes*-like sequences. The 13-month replicate sample from July 2005 showed similar trends, although the

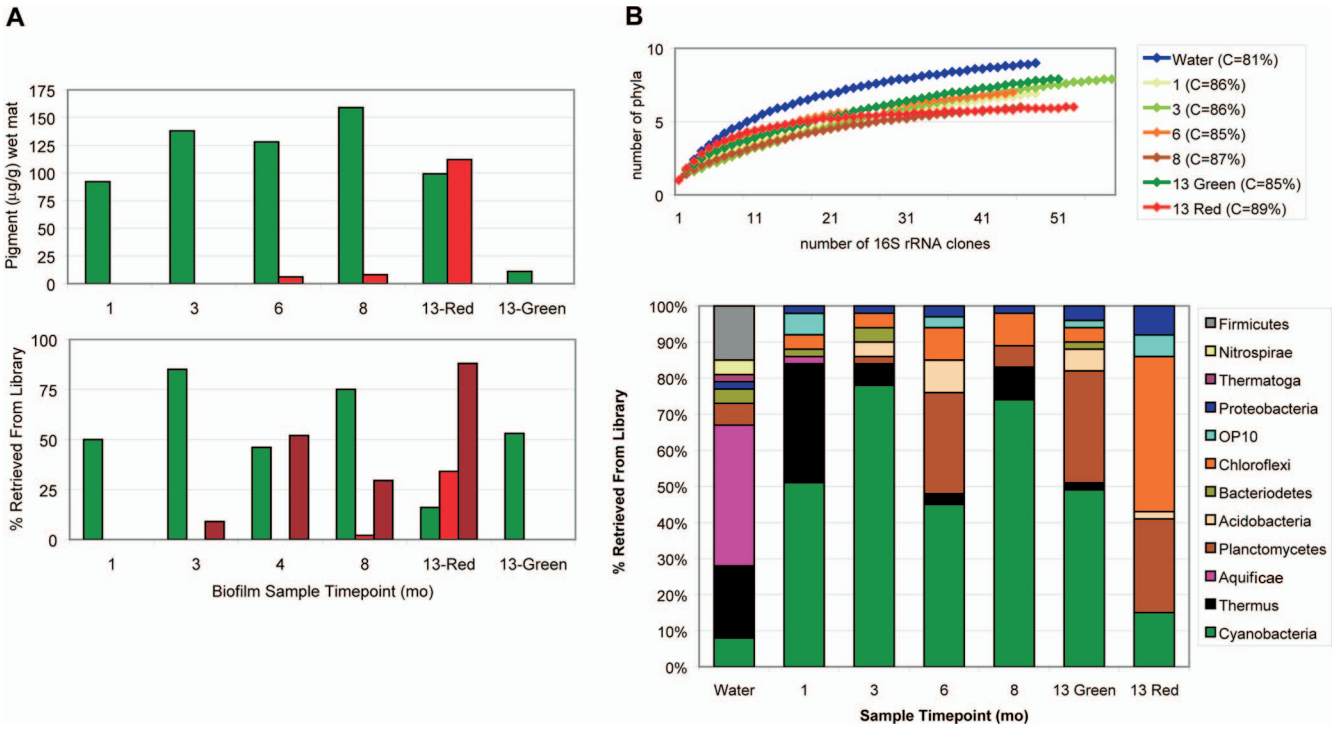

FIG. 2. Color plates showing graphs of pigments and bacterial retrieval rates. (A) Graphs showing pigment analysis and retrieval of phototrophs. In the top graph, total pigment was methanol extracted from each 0.02-g biofilm homogenate (for 1- to 8-month samples) and biofilm layer (for 13-month green and red layers). Absorbance was recorded for green Chl *a* or Bchl *c* pigment peaks (green bars) at 664 to 669 nm and for red pigment Bchl *a* peaks (red bars) at 768 to 771 nm. Pigments were quantified and reported in μg/g wet mat weight. In the bottom graph, the percentages of retrieved sequences for all *Cyanobacteria*-like sequences per general bacterial library (green), the percentages of retrieved sequences for all red *Chloroflexi*-like sequences per *Chloroflexi* library (maroon), and the percentages of retrieved sequences for all red *Chloroflexi*-like sequences per general bacterial library (red) are plotted over time. (B) Graph showing general-bacterial-library analysis, with rarefaction and abundance data. In the top graph, rarefaction curves for total bacterial phyla are shown for each of the assessed water and biofilm samples; coverage values (C) for each sample are indicated in the legend. In the bottom graph, the percentages of retrieved sequences for all phyla from general bacterial libraries are shown for water and biofilm samples over time; each phylum has been color coded according to the key shown adjacent to the graph.

analyzed libraries were not as large (data not shown). Finally, the major sequence types retrieved from filtered water biomass included *Aquificae*, *Deinococcus-Thermus*, and *Firmicutes* (Table 2 and Fig. 2B).

Because *Cyanobacteria*-like sequences were the most readily retrieved sequence type from general bacterial libraries for all biofilm samples in this study, we further categorized each of these 165 clones by using BLAST information. Specifically, we selected 17 clones that represented observed diversity among the five major cyanobacterial groups shown in Table 3; these included four *Cyanobacteria* subsections (*Chroococcales*, *Oscillatoriales*, *Nostocales*, and *Stigonematales*) as well as OS types I and P, both uncultured *Synechococcus* variants despite genetic similarity to members of the *Oscillatoriales* (17, 53, 56). Abundance data for *Cyanobacteria*-like representatives are also summarized in Table 3. At both 1 (June 2004) and 3 (September 2004) months, the most abundant *Cyanobacteria* types were most similar to other hot spring *Synechococcus* isolates from Yellowstone National Park. At 6 months (December 2006), the most abundant cyanobacterial types were most similar to OS types I and P, both from Yellowstone hot springs. At 8 months (February 2007), the most abundant cyanobacterial types were most similar to OS type I and *Fischerella* sequences from other hot springs around the world. At 13 months (July 2007), the most abundant cyanobacterial types in the green layer were most similar to hot spring *Synechococcus* isolates from Yellowstone, OS type I, and *Fischerella* isolates; the 13-month replicate sample from July 2005 showed similar trends, although the analyzed libraries were not as large (data not shown). Finally, *Cyanobacteria*-like sequence types retrieved from filtered water biomass were most similar to hot spring *Synechococcus* isolates from Yellowstone as well as OS types I and P.

***Chloroflexi* library analysis and community composition.** Given our long-term interest in characterizing *Chloroflexi* ecology and evolution, we also applied a *Chloroflexi*-targeted amplification strategy to biofilm samples over time and filtered water biomass. All 208 *Chloroflexi* sequences could be placed into one of five representative groups, based on BLAST hits for the genus with the most-frequently cultured isolates: *Chloroflexus*, *Oscillochloris*, *Heliothrix*, *Roseiflexus*, or *Thermomicrobium* (Table 4). Abundance data for *Chloroflexi* representatives are also summarized in Table 4. Given our interest in understanding the specific dynamics of *Roseiflexus*-like sequences, we plotted the retrieval for just these groups in Fig. 2A. As shown in Table 4, retrieved *Chloroflexi* from the 1-month (June 2004) and 3-month (September 2004) biofilms were dominated by

TABLE 2. BLAST similarity and abundance data for general bacterial libraries

Top phylum[a]	Representative (GenBank accession no.)	Closest cultured hit (GenBank accession no.)	Scores (%)[c]	% of samples retrieved[e]						
				1 mo	3 mo	6 mo	8 mo	13 mo (G)	13 mo (R)	Water
Aquificae	FwatB62 (FJ206275)	*Thermocrinis* (AJ320219)	96, 98	2						39
Acidobacteria	JulGB39 (FJ206621)	*Chloracidobacterium* (EF531339)	96, 99					4		
	Srod37 (FJ206368)	*Acetobacteraceae* (AM749787)	91, 91		4	9		2	2	
Bacteroidetes	JrodB9 (FJ206442)	*Rhodothermus* (AB267450)	79, 99	2	2					2
	JulGB23A (FJ206643)	*Chloroherpeton* (CP001100)	86, 99		2			2		
	FwatB7 (FJ206241)	*Marinicola* (AY739663)	86, 99							2
Chloroflexi	JulRB68 (FJ206533)	*Roseiflexus* (CP000686)	99, 99				2		32	
	JrodB5 (FJ206439)	*Chloroflexus* (AJ308498)	98, 97	4	2	4.5	4		2.5	
	JulRB129 (FJ206567)	*Oscillochloris* (AF093427)	91, 99					2	2.5	
	JulRB37 (FJ206528)	None; all uncultured (top, EF205466)	94, 97		2	4.5	4	2	6	
Cyanobacteria	*[b]	*[b]	*[b]	51	78	45	74	49	15	8
Deinococcus-Thermus	FwatB66 (FJ206279)	*Thermus* (L09663)	96, 98	33	6	3	8	2		20
Firmicutes	FwatB32 (FJ206252)	*Bacillus* (AB330404)	97, 100							8
	FwatB39 (FJ206258)	*Clostridium* (AM261414)	97, 98							5
	FwatB45 (FJ206261)	*Dehalobacter* (DQ777749)	93, 100							2
Nitrospirae	FwatB36 (FJ206255)	None; all uncultured (top, DQ337083)	90, 100							4
OP10	JulRB138 (FJ206572)	None; all uncultured (top, EF205567)	99, 97	6		3		2	6	
Planctomycetes	FwatB58 (FJ206271)	None; all uncultured (top, EU135234)[d]	90, 94		2	28	6	31	22	6
	JulGB15A (FJ206649)	*Isosphaera* (X64372)	96, 96						4	
Proteobacteria	JrodB56A (FJ206475)	*Alysiosphaera* (AY428766)	88, 100	2				2	6	
	JulRB120 (FJ206561)	*Sandaracinobacter* (Y10678)	92, 99			3	2	2	2	
	SrodB13 (FJ206355)	*Polyangium* (AJ233943)	85, 99		2					
	FwatB17 (FJ206246)	*Pantoea* (AF227832)	97, 91							2
Thermatoga	FwatB72 (FJ206283)	*Fervidobacterium* (EF222229)	97, 100							2

[a] Based on the similarity data output from BLAST and RDP classifier searches. We assigned an OTU to a division if it exhibited >75% sequence similarity to a cultured representative.
[b] These results are given in Table 3.
[c] The first and second scores are BLAST maximum identity percentages and query coverage percentages, respectively.
[d] Top hits were typically uncultured soil isolates (as represented here), but hot spring and/or *Gemmata*-like isolates were often obtained or observed among secondary hits.
[e] G, green layer; R, red layer.

Chloroflexus-like sequences. Retrieved *Chloroflexi* from the 6-month (December 2006) and 8-month (February 2007) biofilms included mixtures of *Chloroflexus*- and *Roseiflexus*-like sequences. Retrieved *Chloroflexi* from the 13-month (July 2007) red layer were dominated by *Roseiflexus*-like sequences; the 13-month replicate sample from July 2005 showed similar trends, although the analyzed libraries were not as large (data not shown). Finally, retrieved *Chloroflexi* from filtered water biomass included sequence types that were similar to *Chloroflexus*, *Heliothrix*, *Roseiflexus*, and *Thermomicrobium* isolates (Table 4).

Because we were interested in the phylogenetic relationships between *Chloroflexi* during biofilm formation and development, we analyzed representative *Chloroflexi* from each sampling time point (Fig. 3). The *Chloroflexus*-like representatives from all time points formed a well-defined clade including *Chloroflexus* strain 396 (AJ308498) (35). The *Roseiflexus*-like representatives from all time points formed a well-defined clade with *Roseiflexus* strain RS-1 (unpublished). *Thermomicrobium*-like representatives formed a well-defined clade including *Thermomicrobium roseum* (M34115) (37). The *Heliothrix*-like representative was most closely related to *Heliothrix oregonensis* (42). The *Oscillochloris*-like representative was a member of the clade that included green *Chloroflexi* but was not in the cluster that included *Oscillochloris* controls.

DISCUSSION

Our laboratory's primary interest lies in understanding the diversity and ecology of red filamentous *Chloroflexi* containing only Bchl *a* and related to *Roseiflexus castenholzii* (24) and

TABLE 3. BLAST similarity and abundance data for *Cyanobacteria* libraries

Top subsection (genus or variant)[a]	Representative (GenBank accession no.)	GenBank descriptor[b] (accession no.)	Source[c]	Scores (%)[d]	% of samples retrieved					
					1 mo	3 mo	6 mo	8 mo	13 mo (G[e])	Water
Chroococcales (*Synechococcus*- or *Thermosynechococcus*-like)										
	JrodB13 (FJ206445)	*Synechococcus* strain JA-3-3Ab (CP000239)	Octopus HS*	96, 100	60	30.5	15		4	
	JrodB20 (FJ206451)	UC cyanobacterium (AF505962)	Heart HS*	98, 99	36	30.5	5			
	SrodB24A (FJ206384)	*Synechococcus* strain TS-15 (AY884054)	Octopus HS*	99, 99	4	4.5			4	
	FwatB59 (FJ206272)	*Synechococcus* strain C9 (AF132773)	Octopus HS*	98, 100			5	8.5	31	25
	DrodB46 (FJ206786)	*Thermosynechococcus elongatus* (BA000039)	Japan HS	92, 100			5			
Chroococcales (OS type I- and P-like)[f]										
	SrodB47A (FJ206399)	UC cyanobacterium OS-P (L35331)	Octopus HS*	98, 99		11	15		4	50
	SrodB41A (FJ206395)	Cyanobacterium OS-I (L04709)	Octopus HS*	98, 98		6				25
	SrodB27 (FJ206361)	UC cyanobacterium (EF429514)	Philippines HS	98, 97		13	30	52	15	
	SrodB35A (FJ206391)	UC cyanobacterium (EF126240)	Indonesia mat	96, 99		4.5	5		4	
Oscillatoriales (*Leptolyngbya*-like)										
	JulGB7 (FJ206608)	*Leptolyngbya* strain Greenland_10 (DQ431005)	Greenland HS	95, 98					7	
	DrodB12 (FJ206763)	*Leptolyngbya badia* (EF429297)	alpine seeps	94, 96			5			
	JulGB8 (FJ206609)	*Leptolyngbya* strain NB2a-c2 (EU528665)	not stated	93, 89					4	
Nostocales (*Anabaena*- or *Calothrix*-like)										
	DrodB12A (FJ206803)	UC bacterium (EF467518)	cave biofilm	95, 94			5			
	DrodB50 (FJ206790)	UC cyanobacterium (AY862011)	Queens HS*	96, 92			10			
Stigonematales (*Fischerella*-like)										
	FrodB34 (FJ206706)	*Fischerella* strain MV9 (DQ786169)	Costa Rica HS	99, 99				11	4	
	FrodB21 (FJ206699)	UC bacterium (AF407696)	Australia HS	98, 97				20	23	
	FrodB68 (FJ206726)	UC cyanobacterium (DQ471445)	Jordan HS	94, 98				8.5		

[a] Based on similarity data output from BLAST searches.
[b] UC, uncultured.
[c] HS, hot spring; *, Yellowstone National Park.
[d] The first and second scores are BLAST maximum identity percentages and query coverage percentages, respectively.
[e] G, green layer.
[f] OS type I and P-like sequences were given their own category because they represent uncultured *Synechococcus* variants, despite genetic similarity to members of the *Oscillatoriales* (17, 53, 56).

Roseiflexus strain RS-1 (unpublished). In this study, we tested hypotheses about biofilm development downstream from previously described mat communities at Fairy Geyser (41.3 to 43.5°C), which contain both surface layers of green *Cyanobacteria* and underlayers of *Roseiflexus*-like *Chloroflexi* (5, 6). In experiments for addressing our primary hypothesis, we observed that multilayered biofilms readily established on partially submerged sterile glass rods in the geothermal runoff (Fig. 1). During the course of this study, we observed a gradual transition in biofilm formation that initiated below the water surface (60 to 70°C) but then expanded to the cooler (38.8°C) glass substratum above the water (Fig. 1). Although we mea-

TABLE 4. BLAST similarity and abundance data for *Chloroflexi* libraries

Top genus[a] and representative (GenBank accession no.)	GenBank descriptor[b] (accession no.)	Scores (%)[c]	% of samples retrieved					
			1 mo	3 mo	6 mo	8 mo	13 mo (R[d])	Water
Chloroflexus-like	*Chloroflexus* strain 396-1 (AJ308498)		100	85	48	70.5	10	73
JrodW5 (FJ206407)		98, 99						
SrodW12 (FJ206319)		98, 99						
DrodW3 (FJ206738)		98, 99						
FrodW31 (FJ206667)		98, 99						
JulRW41 (FJ206498)		98, 99						
FwatW11 (FJ206291)		98, 99						
Oscillochloris-like JulRW58 (FJ206511)	UC bacterium (AY349542)	98, 98					2	
Heliothrix-like FwatW29A (FJ206307)	UC *Chloroflexi* (AY184449)	97, 99						4
Roseiflexus-like	*Roseiflexus* strain RS-1 (CP000686)			9	52	29.5	88	11.5
SrodW25A (FJ206336)		99, 99						
DrodW16 (FJ206745)		98, 99						
FrodW4 (FJ206656)		98, 99						
JulRW5 (FJ206486)		98, 99						
FwatW1 (FJ206286)		99, 99						
Thermomicrobium-like	*T. roseum* (M34115)			6				11.5
SrodW7A (FJ206340)		96, 99						
FwatW13 (FJ206292)		99, 99						

[a] Based on the similarity data output from BLAST searches.
[b] UC, uncultured.
[c] The first and second scores are BLAST maximum identity percentages and query coverage percentages, respectively.
[d] R, red layer.

sured some differences in temperature and aqueous sulfur concentration at the vent source and experimental site (Table 1), variations were within the range that we have observed over time at Fairy Geyser (4, 5).

In terms of successional events involving *Chloroflexi*, the first evidence for *Roseiflexus*-like *Chloroflexi* determined by more than one method occurred after a *Cyanobacteria*-dominated biofilm had developed at and above the air-water interface. Between 6 and 8 months (December 2006 to February 2007), *Roseiflexus*-like sequence types were abundant in *Chloroflexi* libraries (Table 4) and retrieved for the first time in general bacterial libraries (Table 2 and Fig. 2). Concurrently, Bchl *a* pigment signatures were also first detected (Fig. 2), along with red filamentous microcolonies (Fig. 1). By 13 months, a visibly distinct red underlayer had fully formed (Fig. 1), with *Roseiflexus*-like sequences representing the most-prevalent types in both libraries assessed (Tables 2 and 4 and Fig. 2). Supporting these trends, we detected the highest levels of Bchl *a* (Fig. 2) and observed microscopic populations dominated by red filamentous cells (Fig. 1).

Phylogenetically, the relationships among *Roseiflexus*-like sequence representatives and *Roseiflexus* strain RS-1 (unpublished) were largely unresolved (Fig. 3). However, Ferris et al. observed at least two *Roseiflexus*-like denaturing gradient gel electrophoresis bands that shifted as a function of time and temperature in the mat community at Octopus Spring (16). Likewise, studies in our laboratory have demonstrated that Red Layer Microbial Observatory communities show site-specific genetic variants related to *Roseiflexus* (5). Given that these previous reports involved established, complex communities, we speculate that water dispersal and early biofilm formation events define a founder effect; consequently, comparable red *Chloroflexi* variants would be observed given more time following red-layer community development. In contrast with *Roseiflexus*-like sequence types, *Chloroflexus*-like sequences were more genetically variable. Notably, *Chloroflexus*-like sequences from early, higher-temperature samples and geothermal runoff water samples formed a distinct subcluster relative to those retrieved from later, lower-temperature biofilm samples (Fig. 3). Given the importance of temperature-driven selection cyanobacterial systems (1, 31), it seems appropriate to speculate that temperature variants also exist among *Chloroflexus* isolates. In addition, we detected some low-abundance representatives that were similar to those of genera typically not associated with these mat systems (36), including *Oscillochloris* (28) and *Heliothrix* (41, 42) (Table 4 and Fig. 3). Given that Nübel et al. have also observed and suggested that there is greater genetic variation among phototrophic *Chloroflexi* in what have historically been considered *Chloroflexus*-dominated mats (36), further studies that describe and cultivate green *Chloroflexi* are warranted.

In terms of our third hypothesis, *Roseiflexus*-like sequences were retrieved from Fairy Geyser vent water, but only using a *Chloroflexi*-targeted amplification approach (Table 4 and Fig. 2). Similar low-abundance *Chloroflexi*-like sequences were also reported to occur in source water at Angel Terrace in Yellowstone National Park (18). Thus, while it seems that geothermal runoff may provide a dispersal mechanism for red *Chloroflexi*, these phototrophs do not represent an abundant sequence type in the water. Moreover, the relative proportions of bacterial sequence types retrieved from the source water did not resemble those retrieved from any biofilm sample (Fig. 2), suggesting that the rod substratum offers a different set of conditions that selects for a community of bacteria different from those inhabiting the bulk aqueous phase. However, the source water con-

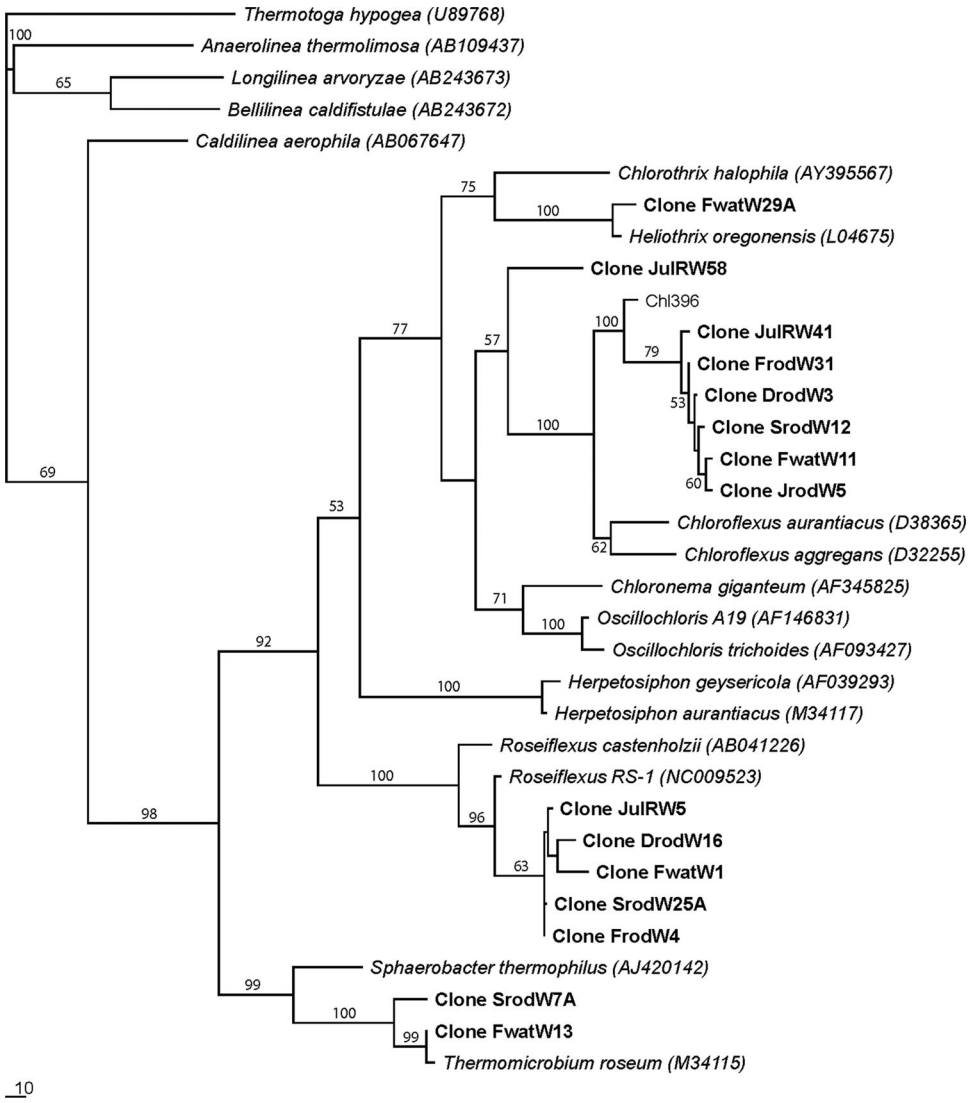

FIG. 3. Maximum parsimony tree of *Chloroflexi* library clones. Known *Chloroflexi* are indicated in italics, with the GenBank accession numbers in parentheses. Representative water and biofilm clones are signified by bold clone names and numbers (Table 4). The bar represents 10 nucleotide changes. In this analysis, there were 1,000 total characters, and 370 were usable for parsimony analysis. A total of 100 bootstrap replicates were performed, and the bootstrap values are indicated (those that were <50% are not shown).

tained many of the same groups of organisms found at different times in the biofilm (e.g., *Aquificae*, *Deinococcus-Thermus*, *Cyanobacteria*, and *Planctomycetes*), suggesting that source water serves as an inoculum for the biofilm community that developed on the rods. Indeed, although our initial interest for this project was *Chloroflexi*, many results provide insight about diversity, population dynamics, and dispersal issues for other key groups of microorganisms. Given these trends, the rest of this discussion will focus on the dynamics of the following microbial groups, with an emphasis on the issues described above: *Cyanobacteria* and high-abundance sequences (>10% in a given library) resembling those for *Aquificae*, *Deinococcus-Thermus*, and *Planctomycetes*.

***Cyanobacteria* trends, diversity, and dispersal.** *Cyanobacteria*-like sequences were the most prevalent sequence type retrieved from all biofilms (Tables 2 and 3 and Fig. 2), consistent with many well-studied Yellowstone communities (53) as well as extreme community habitats worldwide (38). In this study, we were able to correlate the microscopic emergence of filamentous forms (Fig. 1) with molecular data over time (Table 3). Given that all *Cyanobacteria* express common Chl *a* pigment (in methanol extracts, the absorbance peaks of which overlap with green Bchl *c*, also expressed by green *Chloroflexi*), pigment data were informative only in terms of general trends. Notably, green pigment trends followed *Cyanobacteria*-like retrieval data at all time points (Tables 2 and 3 and Fig. 2), with notable seasonal shifts in winter. In the seasonal study at Octopus Spring by Ferris et al., *Cyanobacteria*-like bands showed some similar trends (16). Consequently, additional studies during this seldom-monitored season are warranted.

Over the course of this study, changes in retrieved *Cyanobacteria*-like sequences suggested a shift from a higher-temperature

community characterized by *Synechococcus*-like members to a lower-temperature community that contained both unicellular forms and heterocyst-containing filaments (Table 3 and Fig. 1). The earliest (1-month) sample contained *Synechococcus*-like sequences related to higher-temperature isolates from Octopus Spring (51 to 61°C) (1) and Heart Pool (56.5 to 58.3°C) (39) (Table 3). This finding is consistent with the fact that these earliest biofilms were located on the hotter, submerged portion of the rods. All samples from later time points contained increasing numbers of *Cyanobacteria* representatives, with biofilms accumulating at and above the cooler air-water interface. Although most *Cyanobacteria*-like sequence types from the 3-month sample were most similar to the above-mentioned *Synechococcus* strains, nearly the balance were most similar to OS types I and P, both isolated from lower-temperature (50 to 55°C) regions of Octopus Spring (17, 56) (Table 3), consistent with the fact that more observed biofilm was located at the cooler air-water interface. Although such temperature differences may seem subtle, temperature-based studies using *Synechococcus* models have demonstrated that genetic variants are selected by comparable temperature zones (1, 31).

Winter sample (6- to 8-month) *Cyanobacteria* representatives built on previously described *Synechococcus*-like sequences, adding lower-temperature representatives similar to *Synechococcus* strain C9 (from Octopus Spring, 50 to 55°C) (17) and *Thermosynechococcus* isolates (from Beppu Hot Springs in Japan, 55°C) (34) (Table 3). Although biofilm samples continued to contain unicellular *Cyanobacteria*, we also observed filamentous forms for the first time (Fig. 1), correlating with the emergence of sequence types that were most similar to filamentous *Oscillatoriales* and *Nostocales*. Mixtures of unicellular forms and filaments continued through this study, with *Fischerella*-like sequences appearing after 8 months (Table 3). *Fischerella* isolates, which generally grow at temperatures below 57°C, have also been shown to exhibit geographic clustering and site-specific variation (32, 33).

Consistent with other studies of erupting vent water in Yellowstone (18), levels of *Cyanobacteria*-like sequence types in geothermal runoff were low (Fig. 2 and Table 3), similar to *Synechococcus* strain C9 (17, 50) and OS types I and P (17, 53, 56) (Table 3). Although diverse cell types were observed in filtered water biomass samples, microscopic assessments of these samples were not informative, because we observed no autofluorescing, Chl *a*-containing cell types (Fig. 1), perhaps because temperatures are too hot for *Cyanobacteria* to be metabolically active during hypothesized runoff transport. That we were unable to account for some cyanobacterial groups in Fairy Geyser geothermal water suggests one of two possibilities: (i) unrepresented genera are present, but in such limited numbers that our use of bacterial primers for population screening were inadequate; or (ii) these organisms were transported to the biofilm by alternative means. *Fischerella* isolates have, for example, shown tolerance for freezing and desiccation, suggesting that airborne dispersal is responsible for its widespread distribution (32).

Diversity of chemotroph-like bacteria. Although the majority of sequences retrieved in this study represented photosynthetic *Cyanobacteria* and *Chloroflexi* with diagnostic features that we could correlate with pigment and microscopic data, we retrieved many sequence types that were similar to other phyla. For the purposes of this discussion, we will be emphasizing the following groups that were retrieved in high abundance (>10% in a given library) during at least one sampling time or place: *Aquificae* (all *Thermocrinis*-like), *Deinococcus-Thermus* (all *Thermus*-like), and *Planctomycetes* (all most similar to uncultured sequence types) (Table 2 and Fig. 2).

Although *Thermocrinis*-like sequences were most readily retrieved from geothermal water, they were not retrieved from any biofilm sample except that at 1 month (June 2004) (Table 2). All these sequence types were most similar to *Thermocrinis* strain P2L2B, isolated from a hot spring (65°C) in Yellowstone's Heart Lake Geyser Basin, although typical streaming filaments were not reported and this site represents a low in the reported temperature range for this genus (65 to 96°C) (13). *Aquifex*-like sequences were also the most retrieved sequence type in two other studies of geothermal water, including that from Angel Terrace (18) and borehole geothermal fluid from Iceland (18, 30). *Thermus*-like sequences were the second most prevalent type retrieved from geothermal water (21%) and the 1-month (June 2004) biofilm sample (34%). All biofilm-derived sequence types were most similar to *Thermus aquaticus* YT-1, isolated from a hot spring (70°C) in Yellowstone's Upper Geyser Basin, a high temperature in the 50 to 70°C range reported for this group (46, 57). Because the temperatures at the submerged portion of the rods (60 to 70°C) overlapped well with this range, we were not surprised that *Thermus*-like organisms, given retrieval from geothermal water, were present in early biofilms and maintained representation through 8 months (February 2006). Given that *Thermus* isolates grow chemoheterotrophically (46, 57) and *Thermocrinis* isolates grow chemolithotrophically (13), it seems likely that *Thermus* isolates would be more selected in the context of a complex community biofilm with fixed carbon sources.

In contrast with *Thermus*- and *Thermocrinis*-like sequences, retrieved *Planctomycetes*-like representatives were more prevalent only during later biofilm time points. In contrast with our predictions, the majority of these sequence types were not most similar to *Isosphaera pallida*, isolated from comparable alkaline hot spring communities (41 to 55°C) in Oregon (22). Rather, they were most similar to uncultured sequence types from a diverse array of thermal and nonthermal habitats, including Obsidian Pool in Yellowstone (26). RDP classifier and preliminary phylogenetic analyses (data not shown) suggested that our uncultured *Planctomycetes*-like representative is a member of the *Gemmata* group (21). Both *Gemmata obscuriglobus* (21) and our uncultured *Planctomycetes*-like representative show moderately high G+C contents (57 and 60%, respectively) in the 135- to 228-bp region of the 16S gene (based on *E. coli* 16S structure) (23), which is similar to that of *Isosphaera pallida*. This is also consistent with previous findings indicating that *Gemmata* and *Isosphaera* isolates are the most closely related phylogenetic groups in the *Planctomycetes* phylum (14, 21). In a *Planctomycetes*-focused study at Zodletone Spring, a mesophilic sulfide spring in Oklahoma (14), Elshahed et al. noted that the number of cultured *Planctomycetes* isolates represents a minor fraction of *Planctomycetes*-like 16S rRNA gene sequences available in the GenBank database; likewise, we found over 3,000 by using the search phrase "uncultured *Planctomycetes*," with most either unpublished or secondary com-

ponents of broad bacterial diversity studies like this project. Consequently, despite some synthetic analyses of this phylum (14, 20, 21, 51, 54), additional efforts should be made to cultivate what appear to be novel *Planctomycetes* in Yellowstone hot spring systems and beyond.

Conclusion. In this study of biofilm development over time in a geothermal environment, we have demonstrated that multilayered photosynthetic biofilms can be grown at the air-water interface by using simple artificial substrata suspended in flowing geyser runoff. The temporal pattern for biofilm development in these communities involved the primary establishment of autotrophic *Cyanobacteria*, followed by the appearance of reddish orange microcolonies and *Roseiflexus*-like *Chloroflexi* sequences, cell types, and photopigments after 6 to 8 months. Given that *Roseiflexus castenholzii* and red-layer-associated relatives are photoheterotrophs (6, 24), this finding is consistent with their appearance after *Cyanobacteria* have been established and are providing fixed carbon sources. Given that *Roseiflexus*-like *Chloroflexi* from Fairy Geyser have eluded isolation in pure culture (6, 29), this substrate-based approach could provide a tool for studying and propagating these phototrophs without heavily impacting upon natural splash mats. A second temporal pattern observed in these communities involved *Cyanobacteria* shifting from a more homogeneous, higher-temperature *Synechococcus*-like community to a more diverse, lower-temperature community that contained both unicellular forms and heterocyst-containing filaments. Although we observed some trends in terms of *Thermocrinis*- and *Thermus*-like sequences, both retrieved from higher-temperature geothermal water and early submerged biofilms, the numbers of uncultured *Planctomycetes*-like sequences thriving in established air-water interface biofilms were most intriguing to us, given our specific goal of understanding red-layer communities. Finally, our assessment of biomass in geothermal water at this site showed that *Roseiflexus*-like *Chloroflexi* sequences are present, suggesting that water could provide an inoculum for these and other high-abundance sequence types observed in the biofilms.

ACKNOWLEDGMENTS

This work was supported by an NSF Microbial Observatories/Research at Undergraduate Institute grant (NSF-MO/RUI 0237167).

Undergraduates Peter Williams and Jennifer Esparza (partially supported by an ASM Undergraduate Research Fellowship in 2004) performed some important preliminary work, including troubleshooting water filtration methods and generating preliminary, smaller libraries from the 2004-2005 study, and we thank them for their hard work and important participation in this long-term project. Research technicians Danny Lodge and Kelly Shipley also performed some preliminary studies with early 2004-2005 samples. In terms of research permits and specific requirements for this long-term field study, we greatly appreciate the assistance and support of Yellowstone National Park, in particular Research Permit Facilitator Christie Hendrix. We gratefully acknowledge Matt Kane for encouraging us to initially pursue these studies, Bruce Fouke for his creative and inspiring experiments, Niki Parenteau for her extremely helpful assistance and specific comments about photopigment assessment and biofilm research during the development of this article, Brian Hedlund for his advice about appropriate statistical tools, and Karen Haberman for her conversations about improving our discussion of ecological trends and diversity.

REFERENCES

1. **Allewalt, J. P., M. M. Bateson, N. P. Revsbech, K. Slack, and D. M. Ward.** 2006. Effect of temperature and light on growth of and photosynthesis by *Synechococcus* isolates typical of those predominating in the Octopus Spring microbial mat community of Yellowstone National Park. Appl. Environ. Microbiol. **72:**544–550.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Blank, C. E., S. L. Cady, and N. R. Pace.** 2002. Microbial composition of near-boiling silica-depositing thermal springs throughout Yellowstone National Park. Appl. Environ. Microbiol. **68:**5123–5135.
4. **Boomer, S. M., W. M. Drury, B. E. Dutton, D. P. Lodge, M. S. Boschee, and W. M. Kernan.** 2005. The red layer microbial observatory database: a model for the integration and dissemination of biological and geochemical data via the World Wide Web, p. 235–245. *In* W. P. Inskeep and T. R. McDermott (ed.), Geothermal biology and geochemistry in Yellowstone National Park: Proceedings of the Thermal Biology Institute Workshop, Yellowstone National Park, WY, October 2003. Thermal Biology Institute, Montana State University, Bozeman.
5. **Boomer, S. M., D. P. Lodge, B. E. Dutton, and B. Pierson.** 2002. Molecular characterization of novel red green nonsulfur bacteria from five distinct hot spring communities in Yellowstone National Park. Appl. Environ. Microbiol. **68:**346–355.
6. **Boomer, S. M., B. K. Pierson, R. Austinhirst, and R. W. Castenholz.** 2000. Characterization of novel bacteriochlorophyll-*a*-containing red filaments from alkaline hot springs in Yellowstone National Park. Arch. Microbiol. **174:**152–161.
7. **Bott, T. L., and T. D. Brock.** 1969. Bacterial growth rates above 90 degrees C in Yellowstone hot springs. Science **164:**1411–1412.
8. **Brock, T. D.** 1967. Life at high temperatures. Evolutionary, ecological, and biochemical significance of organisms living in hot springs is discussed. Science **158:**1012–1019.
9. **Cady, S. L., and J. D. Farmer.** 1996. Fossilization processes in siliceous thermal springs: trends in preservation along thermal gradients, p. 150–173. *In* G. R. Bock and J. A. Goode (ed.), Evolution of hydrothermal systems on Earth (and Mars?): Ciba Foundation Symposium 202. John Wiley, Chichester, United Kingdom.
10. **Caldwell, D. E., E. Atuku, D. C. Wilkie, K. P. Wivcharuk, S. Karthikeyan, D. R. Korber, D. F. Schmid, and G. M. Wolfaardt.** 1997. Germ theory vs. community theory in understanding and controlling the proliferation of biofilms. Adv. Dent. Res. **11:**4–13.
11. **Costerton, J. W., K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. **41:**435–464.
12. **Davey, M. E., and A. O'Toole, G.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. **64:**847–867.
13. **Eder, W., and R. Huber.** 2002. New isolates and physiological properties of the *Aquificales* and description of *Thermocrinis albus* sp. nov. Extremophiles **6:**309–318.
14. **Elshahed, M. S., N. H. Youssef, Q. Luo, F. Z. Najar, B. A. Roe, T. M. Sisk, S. I. Buhring, K. U. Hinrichs, and L. R. Krumholz.** 2007. Phylogenetic and metabolic diversity of *Planctomycetes* from anaerobic, sulfide- and sulfur-rich Zodletone Spring, Oklahoma. Appl. Environ. Microbiol. **73:**4707–4716.
15. **Fardeau, M. L., B. Ollivier, B. K. Patel, M. Magot, P. Thomas, A. Rimbault, F. Rocchiccioli, and J. L. Garcia.** 1997. *Thermotoga hypogea* sp. nov., a xylanolytic, thermophilic bacterium from an oil-producing well. Int. J. Syst. Bacteriol. **47:**1013–1019.
16. **Ferris, M. J., S. C. Nold, N. P. Revsbech, and D. M. Ward.** 1997. Population structure and physiological changes within a hot spring microbial mat community following disturbance. Appl. Environ. Microbiol. **63:**1367–1374.
17. **Ferris, M. J., A. L. Ruff-Roberts, E. D. Kopczynski, M. M. Bateson, and D. M. Ward.** 1996. Enrichment culture and microscopy conceal diverse thermophilic *Synechococcus* populations in a single hot spring microbial mat habitat. Appl. Environ. Microbiol. **62:**1045–1050.
18. **Fouke, B. W., G. T. Bonheyo, B. Sanzenbacher, and J. Frias-Lopez.** 2003. Partitioning of bacterial communities between travertine depositional facies at Mammoth Hot Springs, Yellowstone National Park, USA. Can. J. Earth Sci. **40:**1531–1548.
19. **Fouke, B. W., J. D. Farmer, D. J. Des Marais, L. Pratt, N. C. Sturchio, P. C. Burns, and M. K. Discipulo.** 2000. Depositional facies and aqueous-solid geochemistry of travertine-depositing hot springs (Angel Terrace, Mammoth Hot Springs, Yellowstone National Park, USA). J. Sediment Res. A **70:**565–585.
20. **Fuerst, J. A.** 1995. The planctomycetes: emerging models for microbial ecology, evolution and cell biology. Microbiology **141:**1493–1506.
21. **Fuerst, J. A., H. G. Gwilliam, M. Lindsay, A. Lichanska, C. Belcher, J. E. Vickers, and P. Hugenholtz.** 1997. Isolation and molecular identification of planctomycete bacteria from postlarvae of the giant tiger prawn, *Penaeus monodon*. Appl. Environ. Microbiol. **63:**254–262.
22. **Giovannoni, S. J., E. Schabtach, and R. W. Castenholz.** 1987. *Isosphaera pallida*, gen. and comb. nov., a gliding, budding eubacterium from hot springs. Arch. Microbiol. **147:**276–284.
23. **Gutell, R. R., B. Weiser, C. R. Woese, and H. F. Noller.** 1985. Comparative anatomy of 16S-like ribosomal RNA. Prog. Nucleic Acids Res. Mol. Biol. **32:**155–216.

24. **Hanada, S., S. Takaichi, K. Matsuura, and K. Nakamura.** 2002. *Roseiflexus castenholzii* gen. nov., sp. nov., a thermophilic, filamentous, photosynthetic bacterium that lacks chlorosomes. Int. J. Syst. Bacteriol. **52:**187–193.
25. **Huber, T., G. Faulkner, and P. Hugenholtz.** 2004. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics **20:**2317–2319.
26. **Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace.** 1998. Novel division level bacterial diversity in a Yellowstone hot spring. J. Bacteriol. **180:**366–376.
27. **Kandianis, M. T., B. W. Fouke, R. W. Johnson, J. Veysey II, and W. P. Inskeep.** 2007. Microbial biomass: a catalyst for $CaCO_3$ precipitation in advection-dominated transport regimes. Geol. Soc. Am. Bull. **120:**442–450.
28. **Keppen, O. I., T. P. Tourova, B. B. Kuznetsov, R. N. Ivanovsky, and V. M. Gorlenko.** 2000. Proposal of *Oscillochloridaceae* fam. nov. on the basis of a phylogenetic analysis of the filamentous anoxygenic phototrophic bacteria, and emended description of *Oscillochloris* and *Oscillochloris trichoides* in comparison with further new isolates. Int. J. Syst. Evol. Microbiol. **50:**1529–1537.
29. **Manning, T. F.** 2005. Attempts to cultivate red layer bacteria using site-specific water chemistry data. Undergraduate honors thesis. Western Oregon University, Monmouth.
30. **Marteinsson, V. T., S. Hauksdottir, C. F. Hobel, H. Kristmannsdottir, G. O. Hreggvidsson, and J. K. Kristjansson.** 2001. Phylogenetic diversity analysis of subterranean hot springs in Iceland. Appl. Environ. Microbiol. **67:**4242–4248.
31. **Miller, S. R., and R. W. Castenholz.** 2000. Evolution of thermotolerance in hot spring cyanobacteria of the genus *Synechococcus*. Appl. Environ. Microbiol. **66:**4222–4229.
32. **Miller, S. R., R. W. Castenholz, and D. Pedersen.** 2007. Phylogeography of the thermophilic cyanobacterium *Mastigocladus laminosus*. Appl. Environ. Microbiol. **73:**4751–4759.
33. **Miller, S. R., M. D. Purugganan, and S. E. Curtis.** 2006. Molecular population genetics and phenotype diversification of two populations of the thermophilic cyanobacterium *Mastigocladus laminosus*. Appl. Environ. Microbiol. **72:**2793–2800.
34. **Nakamura, Y., T. Kaneko, S. Sato, M. Ikeuchi, H. Katoh, S. Sasamoto, A. Watanabe, M. Iriguchi, K. Kawashima, T. Kimura, Y. Kishida, C. Kiyokawa, M. Kohara, M. Matsumoto, A. Matsuno, N. Nakazaki, S. Shimpo, M. Sugimoto, C. Takeuchi, M. Yamada, and S. Tabata.** 2002. Complete genome structure of the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 (supplement). DNA Res. **9:**135–148.
35. **Nübel, U., M. M. Bateson, M. T. Madigan, M. Kühl, and D. M. Ward.** 2001. Diversity and distribution in hypersaline microbial mats of bacteria related to *Chloroflexus* spp. Appl. Environ. Microbiol. **67:**4365–4371.
36. **Nübel, U., M. M. Bateson, V. Vandieken, A. Wieland, M. Kühl, and D. M. Ward.** 2002. Microscopic examination of distribution and phenotypic properties of phylogenetically diverse *Chloroflexaceae*-related bacteria in hot spring microbial mats. Appl. Environ. Microbiol. **68:**4593–4603.
37. **Oyaizu, H., B. A. Debrunner-Vossbrinck, L. Mandelco, J. A. Studier, and C. R. Woese.** 1987. The green non-sulfur bacteria: a deep branching in the eubacterial line of descent. Syst. Appl. Microbiol. **9:**47–53.
38. **Paerl, H. W., J. L. Pinckney, and T. F. Steppe.** 2000. Cyanobacterial-bacterial mat consortia: examining the functional unit of microbial survival and growth in extreme environments. Environ. Microbiol. **2:**11–26.
39. **Papke, R. T., N. B. Ramsing, M. M. Bateson, and D. M. Ward.** 2003. Geographical isolation in hot spring cyanobacteria. Environ. Microbiol. **5:**650–659.
40. **Pierson, B. K., and R. W. Castenholz.** 1995. Taxonomy and physiology of filamentous anoxygenic phototrophs, p. 31–47. *In* R. E. Blakenship, M. T. Madigan, and C. E. Bauer (ed.), Anoxygenic photosynthetic bacteria. Kluwer Academic Publishers, Dordrecht, The Netherlands.
41. **Pierson, B. K., S. J. Giovannoni, and R. W. Castenholz.** 1984. Physiological ecology of a gliding bacterium containing bacteriochlorophyll *a*. Appl. Environ. Microbiol. **47:**576–584.
42. **Pierson, B. K., S. J. Giovannoni, D. A. Stahl, and R. W. Castenholz.** 1985. *Heliothrix oregonensis*, gen. nov., sp. nov., a phototrophic filamentous gliding bacterium containing bacteriochlorophyll *a*. Arch. Microbiol. **142:**164–167.
43. **Pierson, B. K., A. Oesterle, and G. L. Murphy.** 1987. Pigments, light penetration, and photosynthetic activity in the multi-layered microbial mats of Great Sippewissett Salt Marsh, Massachusetts. FEMS Microbiol. Ecol. **45:**365–376.
44. **Ravenschlag, K., K. Sahm, J. Pernthaler, and R. Amann.** 1999. High bacterial diversity in permanently cold marine sediments. Appl. Environ. Microbiol. **65:**3982–3989.
45. **Reysenbach, A. L., G. S. Wickham, and N. R. Pace.** 1994. Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. Appl. Environ. Microbiol. **60:**2113–2119.
46. **Saul, D. J., A. G. Rodrigo, R. A. Reeves, L. C. Williams, K. M. Borges, H. W. Morgan, and P. L. Bergquist.** 1993. Phylogeny of twenty *Thermus* isolates constructed from 16S rRNA gene sequence data. Int. J. Syst. Bacteriol. **43:**754–760.
47. **Subramaniam, S.** 1998. The Biology Workbench—a seamless database and analysis environment for the biologist. Proteins **32:**1–2.
48. **Swofford, D. L.** 2000. PAUP 4.0b10. Sinauer Associates, Inc., Sunderland, MA.
49. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
50. **Turner, S., K. M. Pryer, V. P. Miao, and J. D. Palmer.** 1999. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J. Eukaryot. Microbiol. **46:**327–338.
51. **Wagner, M., and M. Horn.** 2006. The Planctomycetes, Verrucomicrobia, Chlamydiae and sister phyla comprise a superphylum with biotechnological and medical relevance. Curr. Opin. Biotechnol. **17:**241–249.
52. **Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole.** 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. **73:**5261–5267.
53. **Ward, D. M., M. J. Ferris, S. C. Nold, and M. M. Bateson.** 1998. A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. Microbiol. Mol. Biol. Rev. **62:**1353–1370.
54. **Ward, N., F. A. Rainey, E. Stackebrandt, and H. Schlesner.** 1995. Unraveling the extent of diversity within the order *Planctomycetales*. Appl. Environ. Microbiol. **61:**2270–2275.
55. **Weidler, G. W., M. Dornmayr-Pfaffenhuemer, F. W. Gerbl, W. Heinen, and H. Stan-Lotter.** 2007. Communities of *Archaea* and *Bacteria* in a subsurface radioactive thermal spring in the Austrian Central Alps, and evidence of ammonia-oxidizing *Crenarchaeota*. Appl. Environ. Microbiol. **73:**259–270.
56. **Weller, R., M. M. Bateson, B. K. Heimbuch, E. D. Kopczynski, and D. M. Ward.** 1992. Uncultivated cyanobacteria, *Chloroflexus*-like inhabitants, and spirochete-like inhabitants of a hot spring microbial mat. Appl. Environ. Microbiol. **58:**3964–3969.
57. **Williams, R. A., K. E. Smith, S. G. Welch, and J. Micallef.** 1996. *Thermus oshimai* sp. nov., isolated from hot springs in Portugal, Iceland, and the Azores, and comment on the concept of a limited geographical distribution of *Thermus* species. Int. J. Syst. Bacteriol. **46:**403–408.