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

Sarah M. Boomer, Katherine L. Noll, Gill G. Geesey, and Bryan E. Dutton
Western Oregon University, Department of Biology, Monmouth, Oregon 97361; Quality Schools International, Chengdu, China 610000; and Montana State University, Department of Microbiology, Bozeman, Montana 59717

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 typically consume hydrogen sulfide, 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 geysirite 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 spiculiferous geysirite 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 during 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′58″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 waterline and the point of attachment. We chose glass rods over slides because Owing to environmental resource impact and winter access issues, 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 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) was distributed into 0.2-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 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
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 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

<table>
<thead>
<tr>
<th>Location and parameter</th>
<th>Value for indicated mo and yr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>June 2006</td>
</tr>
<tr>
<td>Fairy vent source</td>
<td></td>
</tr>
<tr>
<td>Mat pH</td>
<td>9.5</td>
</tr>
<tr>
<td>Mat temp (°C)</td>
<td>43.5</td>
</tr>
<tr>
<td>Water pH</td>
<td>8.0</td>
</tr>
<tr>
<td>Water temp (°C)</td>
<td>90</td>
</tr>
<tr>
<td>Sulfide level (mg/liter)</td>
<td>0.04</td>
</tr>
<tr>
<td>Sulfate level (mg/liter)</td>
<td>18.00</td>
</tr>
<tr>
<td>Nitrate level (mg/liter)</td>
<td>5.3</td>
</tr>
<tr>
<td>Biofilm experiment site</td>
<td></td>
</tr>
<tr>
<td>Biofilm pH</td>
<td>Too thin to measure</td>
</tr>
<tr>
<td>Biofilm temp (°C)</td>
<td>Too thin to measure</td>
</tr>
<tr>
<td>Water pH</td>
<td>8.3</td>
</tr>
<tr>
<td>Water temp (°C)</td>
<td>71.1</td>
</tr>
<tr>
<td>Sulfide level (mg/liter)</td>
<td>0.02</td>
</tr>
<tr>
<td>Sulfate level (mg/liter)</td>
<td>13.00</td>
</tr>
<tr>
<td>Nitrate level (mg/liter)</td>
<td>3.20</td>
</tr>
</tbody>
</table>

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
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. 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 hot springs around the world. At 13 months (July 2007), the most abundant cyanobacterial types in the green layer were most similar to other 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 *Thermomicrobiurn* (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
Chloroflexus-like sequences. Retrieved Chloroflexus from the 6-month (December 2006) and 8-month (February 2007) biofilms included mixtures of Chloroflexus- and Roseiflexus-like sequences. Retrieved Chloroflexus 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). Thermomicrobiunm-like representatives formed a well-defined clade including Thermomicrobiunm 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
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 3. BLAST similarity and abundance data for Cyanobacteria libraries

<table>
<thead>
<tr>
<th>Top subsection (genus or variant)*</th>
<th>Representative (GenBank accession no.)</th>
<th>GenBank descriptor* (accession no.)</th>
<th>Source*</th>
<th>Scores (%)</th>
<th>1 mo</th>
<th>3 mo</th>
<th>6 mo</th>
<th>8 mo</th>
<th>13 mo (G')</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chroococcales (Synechococcus- or Thermosynechococcus-like)</td>
<td>JrodB13 (FJ206445)</td>
<td>Synechococcus strain JA-3-3Ab (CP000239)</td>
<td>Octopus HS*</td>
<td>96, 100</td>
<td>60</td>
<td>30.5</td>
<td>15</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>JrodB20 (FJ206451)</td>
<td>UC cyanobacterium (AF505962)</td>
<td>Heart HS*</td>
<td>98, 99</td>
<td>36</td>
<td>30.5</td>
<td>5</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SrodB24A (FJ206384)</td>
<td>Synechococcus strain TS-15 (AY884054)</td>
<td>Octopus HS*</td>
<td>99, 99</td>
<td>4</td>
<td>4.5</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FwatB59 (FJ206272)</td>
<td>Synechococcus strain C9 (AF132773)</td>
<td>Octopus HS*</td>
<td>98, 100</td>
<td>5</td>
<td>8.5</td>
<td>31</td>
<td>25</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DrodB46 (FJ206786)</td>
<td>Thermosynechococcus elongatus (BA000039)</td>
<td>Japan HS</td>
<td>92, 100</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chroococcales (OS type I- and P-like)**</td>
<td>SrodB47A (FJ206399)</td>
<td>UC cyanobacterium OS-P (L53331)</td>
<td>Octopus HS*</td>
<td>98, 99</td>
<td>11</td>
<td>15</td>
<td>4</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SrodB41A (FJ206395)</td>
<td>Cyanobacterium OS-I (L04709)</td>
<td>Octopus HS*</td>
<td>98, 98</td>
<td>6</td>
<td></td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SrodB27 (FJ206361)</td>
<td>UC cyanobacterium (EF429514)</td>
<td>Philippines HS</td>
<td>98, 97</td>
<td>13</td>
<td>30</td>
<td>52</td>
<td>15</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SrodB35A (FJ206391)</td>
<td>UC cyanobacterium (EF126240)</td>
<td>Indonesia mat</td>
<td>96, 99</td>
<td>4.5</td>
<td>5</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oscillatoriales (Leptolyngbya-like)</td>
<td>JulGB7 (FJ206608)</td>
<td>Leptolyngbya strain Greenland_10 (DO431005)</td>
<td>Greenland HS</td>
<td>95, 98</td>
<td></td>
<td></td>
<td></td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DrodB12 (FJ206763)</td>
<td>Leptolyngbya badia (EF429297)</td>
<td>alpine seeps</td>
<td>94, 96</td>
<td></td>
<td></td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>JulGB8 (FJ206609)</td>
<td>Leptolyngbya strain NB2a-c2 (EU528665)</td>
<td>not stated</td>
<td>93, 89</td>
<td></td>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nostocales (Anabaena- or Calothrix-like)</td>
<td>DrodB12A (FJ206803)</td>
<td>UC bacterium (EF467518)</td>
<td>cave biofilm</td>
<td>95, 94</td>
<td></td>
<td></td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DrodB50 (FJ206790)</td>
<td>UC cyanobacterium (AY862011)</td>
<td>Queens HS*</td>
<td>96, 92</td>
<td></td>
<td></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stigonematales (Fischerella-like)</td>
<td>FrodB34 (FJ206706)</td>
<td>Fischerella strain MV9 (DQ786169)</td>
<td>Costa Rica HS</td>
<td>99, 99</td>
<td></td>
<td></td>
<td>11</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FrodB21 (FJ206699)</td>
<td>UC bacterium (AF407096)</td>
<td>Australia HS</td>
<td>98, 97</td>
<td></td>
<td></td>
<td>20</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FrodB68 (FJ206726)</td>
<td>UC cyanobacterium (DQ471445)</td>
<td>Jordan HS</td>
<td>94, 98</td>
<td></td>
<td></td>
<td>8.5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Based on similarity data output from BLAST searches.
** UC, uncultured.
* HS, hot spring; +, Yellowstone National Park.
** The first and second scores are BLAST maximum identity percentages and query coverage percentages, respectively.
** G, green layer.
** 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).
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 Chloroflexus in what have historically been considered Chloroflexus-dominated mats (36), further studies that describe and cultivate green Chloroflexus 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-
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 all 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 phototrophs and heterocyst-containing filaments. Although we observed some trends in terms of *Thermocrinis* and *Thermus*-like sequences, both retrieved from high-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**


PHOTOSYNTHETIC-BIOFILM FORMATION IN SITU


