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Characterization of novel bacteriochlorophyll-*a*-containing red filaments from alkaline hot springs in Yellowstone National Park

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Abstract Novel red, filamentous, gliding bacteria formed deep red layers in several alkaline hot springs in Yellowstone National Park. Filaments contained densely layered intracellular membranes and bacteriochlorophyll *a*. The in vivo absorption spectrum of the red layer filaments was distinct from other phototrophs, with unusual bacteriochlorophyll *a* signature peaks in the near-infrared (IR) region (807 nm and 911 nm). These absorption peaks were similar to the wavelengths penetrating to the red layer of the mats as measured with in situ spectroradiometry. The filaments also demonstrated maximal photosynthetic uptake of radiolabeled carbon sources at these wavelengths. The red layer filaments displayed anoxygenic photoheterotrophy, as evidenced by the specific incorporation of acetate, not bicarbonate, and by the absence of oxygen production. Photoheterotrophy was unaffected by sulfide and oxygen, but was diminished by high-intensity visible light. Near-IR radiation supported photoheterotrophy. Morphologically and spectrally similar filaments were observed in several springs in Yellowstone National Park, including Octopus Spring. Taken together, these data suggest that the red layer filaments are most similar to the photoheterotroph, *Heliothrix oregonensis*. Notable differences include mat position and coloration, absorption spectra, and prominent intracellular membranes.

Key words Photosynthetic bacteria · Microbial mats · Thermophiles · Anoxygenic photosynthesis · Gliding motility · Bacteriochlorophyll · Hot springs · Green non-sulfur bacteria · Photoheterotrophy · Infrared radiation

Abbreviations *Bchl* Bacteriochlorophyll · *Chl* Chlorophyll · *MCLO* Marine *Chloroflexus*-like organism · *RCR* Rabbit Creek Red

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Introduction

Filamentous bacteria containing bacteriochlorophylls (Bchl) exist in several habitats (Pierson and Castenholz 1995). These organisms can be broadly divided into two groups: those with both Bchl *a* and *c* and those with only Bchl *a*. The former group includes the genera *Chloroflexus*, *Chloronema*, and *Oscillochloris*, and the related marine *Chloroflexus*-like organisms (MCLOs) observed in hypersaline mats (Pierson et al. 1994). *Chloroflexus* spp have been observed in hot springs, hypersaline, marine, and freshwater habitats (Pierson and Castenholz 1995). Two species, *Cfl. aurantiacus* and *Cfl. aggregans*, have been cultivated axenically from freshwater thermal environments (Castenholz and Pierson 1995; Hanada et al. 1995; Pierson and Castenholz 1995). *Chloroflexus* spp are typically photoheterotrophs, although some strains can grow photoautotrophically (Giovannoni et al. 1987). *Osc. trichoides* has been isolated from freshwater non-thermal environments and is also a photoheterotroph capable of sulfide-dependent autotrophy (Keppen et al. 1994). Both *Oscillochloris* and *Chloroflexus* species contain chlorosomes located around the periphery of the cell. The MCLOs have not been isolated in pure culture, so less is known about their physiology. They also contain chlorosomes but these are found in the cell interior, lining intracellular proliferations of the cell membrane (Pierson et al. 1994).

Filamentous bacteria containing only Bchl *a* have been observed in but not isolated from freshwater thermal and marine hypersaline environments (Pierson and Castenholz 1995). We previously described the filamentous photo-

heterotrophic Bchl-*a*-containing bacterium, *Heliothrix oregonensis* from hot springs in Oregon (Kahneeta, Warm Springs; Pierson et al. 1984, 1985). This bacterium has not been successfully cultivated in axenic culture, although one successful co-culture with the chemotrophic bacterium, *Isosphaera pallida*, was obtained (Pierson et al. 1984). *Htr. oregonsis* formed a bright orange layer on the surface of mats when exposed to high-intensity light and high oxygen levels; and darkened *Htr. oregonensis* mats were observed to form accumulations of red filaments. Cells of *Htr. oregonsis* contained no distinctive ultrastructural characteristics commonly associated with phototrophic bacteria (Pierson et al. 1984, 1985).

Another filamentous Bchl-*a*-containing phototroph described as a purple bacterium was observed within the sheathed bundles of the cyanobacterium *Microcoleus chthonoplastes* in hypersaline mats (D'Amelio et al. 1987). Relatively little is known about these purple bacteria. Electron microscopy revealed highly stacked intracellular membranes similar in quantity and location within the cells to the membranes described in *Thiocapsa pfennigii* (Drews and Golecki 1995). The cells appeared to be autotrophic, but this was not demonstrated unequivocally (D'Amelio et al. 1987).

We observed unusual filamentous gliding bacteria containing only Bchl *a* that formed distinct deep red or orange layers in several alkaline hot spring mats (30 °C to over 56 °C) in Yellowstone National Park. The red bacteria were first discovered by Castenholz (1984) and have been previously described in meetings and mentioned in reviews: Boomer et al. (1990, 1995); Pierson et al. (1990); Jørgensen et al. (1992); Pierson and Castenholz (1992, 1995); Pierson (1994); Castenholz and Pierson (1995). Prior to this work, the dark red layers observed at the base of many thermal mats were not recognized as filamentous bacteria distinct from *Chloroflexus* spp (Doemel and Brock 1977; Brock 1978). In this study, our primary objective was to describe the structure and ecophysiology of the red filamentous bacteria forming conspicuous deep red layers in selected hot spring microbial mats in Yellowstone National Park. Another objective was to compare these red filamentous undermat bacteria to the orange, filamentous, surface-dwelling *Htr. oregonensis* (Pierson et al. 1984, 1985).

Materials and methods

Microscopy

Light microscopy and infrared (IR) fluorescence microscopy were carried out using a Zeiss Model 16 microscope fitted with an epifluorescence unit and video monitor sensitive to IR (Pierson et al. 1994). Preparation of cells for electron microscopy was modified from D'Amelio et al. (1987). Freshly collected mat was fixed in 2.5% glutaraldehyde in spring water, followed by two 15-min rinses in 0.2 M sodium cacodylate buffer (pH 7.4). Secondary fixation was in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h. Dehydration was conducted in ethanol (50, 70, 90, and 100%). Infiltration with propylene oxide, followed by 50:50 propylene oxide:Spurr's resin, was conducted under vacuum. Samples were embedded in Spurr's resin and polymerized for 12 h at 65–70 °C. Sections were post-stained with uranyl acetate and lead citrate and were viewed on a Zeiss EM109 electron microscope.

Photosynthetic activity

Acetate and bicarbonate labeled with ^{14}C were used for uptake studies (Pierson et al. 1994). All experiments were conducted on freshly collected filaments suspended in spring water. All incubations were in 2-ml screw-cap vials, which were filled to the neck and contained 5 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea to inhibit any contamination by cyanobacteria. Incubations contained final radioactive concentrations of 0.01 μCi [^{14}C]-acetate ml^{-1} or 1.0 μCi [^{14}C]-bicarbonate ml^{-1}. For some experiments, neutralized sodium sulfide was added to a final concentration of 0.5 mM. Vials were incubated for 1 h at 39–42 °C unless otherwise indicated. Samples were incubated at the desired temperatures by submerging vials in the hot spring drainway at the appropriate temperature. Activity was stopped with the addition of 0.1 ml formalin. Samples were refrigerated until analyzed. Samples were incubated in triplicate and data were reported as means with standard deviations.

To test the effect of different lighting conditions, vials were incubated on their side in blackened petri dishes covered with lids fitted with different filters to create the appropriate lighting conditions. For the action spectrum, 10 nm half-maximal band-pass filters were used (Esco Products, Oak Ridge, N.J.). Wavelengths were 640, 670, 710, 740, 790, 840, 910, 950, 1020, and 1060 nm. For in situ incubation, light was collimated with 15-cm chimneys before it passed through the filter. Transmission through the filter was measured with a spectroradiometer (LI-1800; Li-COR, Lincoln, Neb.) under identical conditions to the sample incubation and was integrated to measure the total downward irradiance impinging on the vials. For light-intensity uptake experiments, neutral-density vinyl film was used (Universal Plastics, Portland, Ore.). For IR-uptake experiments, Kodak Wratten 88A gelatin filters sealed in glass plates as previously described were used (Pierson et al. 1994). Darkened conditions were obtained by incubating the vials in totally blackened petri dishes submerged in the hot springs. Samples incubated within the mat were buried in the mat at the appropriate depth. Because of the low light levels within the mat, samples were incubated for 4.75 h in this experiment.

Oxic versus anoxic conditions were created by continuously sparging vials with argon or air during incubation. Vials were fitted with rubber serum caps and gas was delivered through a syringe needle to the base of the incubation sample. Excess gas escaped through a second needle punctured through the cap into the headspace. Samples were incubated at 50% maximum solar irradiance.

Filaments were collected by vacuum filtration onto 0.22-μm Metricel membrane filters (Gelman Sciences, Ann Arbor, Mich.) and processed for liquid scintillation counting as previously described (Pierson et al. 1994). Cells were washed with 0.10 M cold sodium acetate and distilled water. Bicarbonate uptake samples were washed with 1.0 N HCl. Samples were counted in a Beckman, LS 3133T liquid scintillation counter using Biofluor (NEN Research Products, Dupont).

Spectral irradiance

Downward spectral irradiance as a function of depth in the mat was measured using a LI-1800 spectroradiometer (LI-COR, Lincoln, Neb.) with a fiber optic tip (Pierson et al. 1990). Downward irradiance penetrating below specific colored layers of mat were obtained by layering the appropriate sectioned mat on top of the LI-COR remote cosine receptor (LI-1800-11).

Pigment and protein analysis

In vivo absorption spectra of pigments were obtained from sonicated suspensions of filaments or whole mat in buffer as previ-

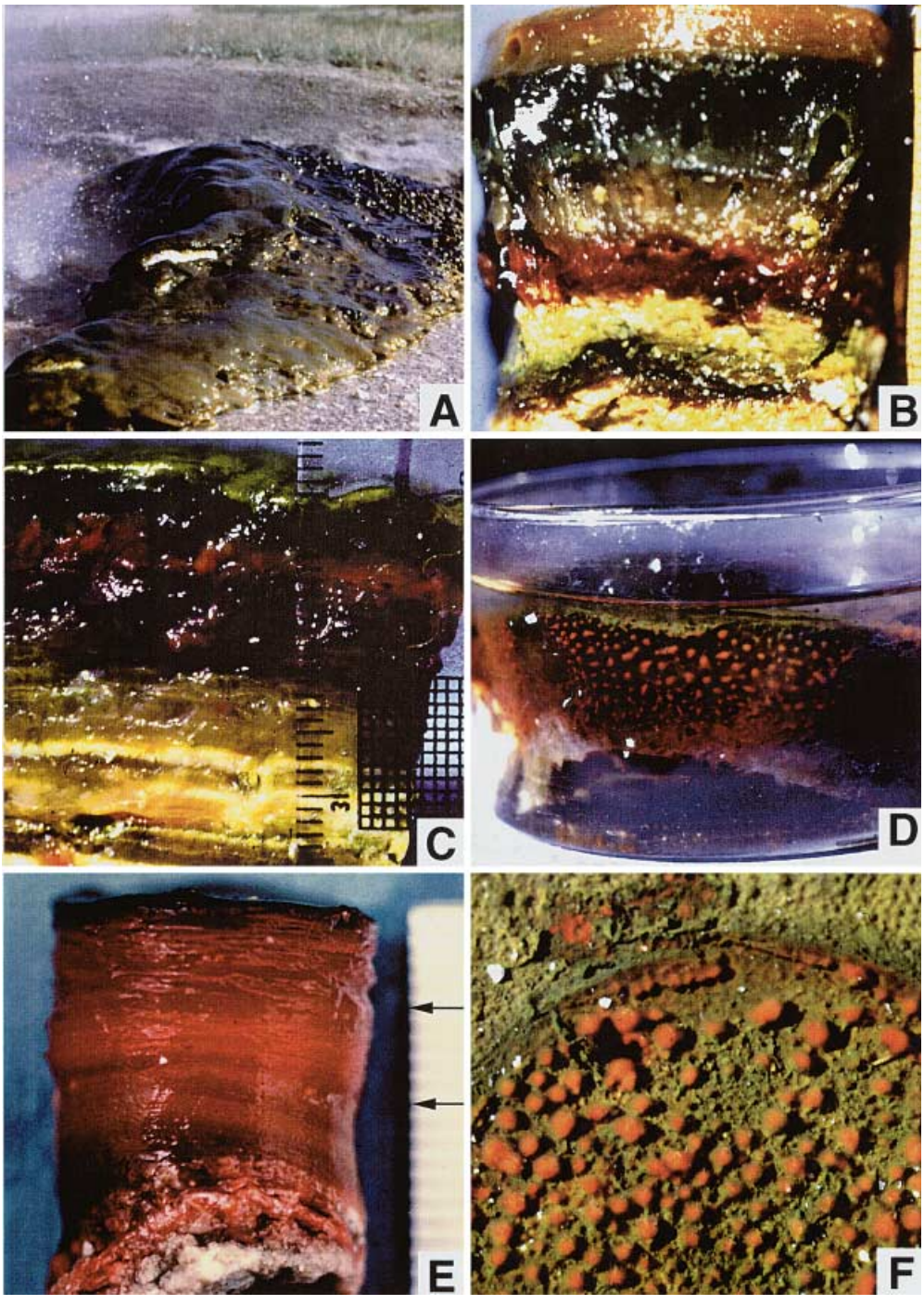

Fig. 1 **A** Rabbit Creek Spouter and mat, Yellowstone National Park. **B** Rabbit Creek Spouter mat core as measured in 1981. The smallest unit of the adjacent *ruler* is 1 mm. See text for description of layers. **C** Rabbit Creek Spouter mat section as measured in 1987. The smallest unit of the adjacent *ruler* is 1 mm. **D** Excised section of Rabbit Creek Spouter mat that displays orange "puffs". **E** Octopus Spring mat core, Yellowstone National Park. The distance between the *arrows* is 5 mm. **F** Octopus Spring mat surface after darkening for 24 h reveals orange "puffs"

ously described (Pierson et al. 1987, 1990, 1994). Methanol spectra and protein values were determined as previously described (Pierson et al. 1987, 1994). All spectra were recorded on a Cary 2300 UV/Vis/near-IR spectrophotometer (Varian-Techtron, Mulgrave, Australia).

Microelectrode measurements

Depth profiles of oxygen concentration and rates of oxygen production were measured with glass microelectrodes (Revsbech and Ward 1984).

Results and Discussion

Rabbit Creek Spouter mat description

Rabbit Creek Spouter was a constantly splashing miniature geyser that spouted water approximately 30–40 cm in height. The source water was 83 °C and lacked sulfide. A thick gelatinous splash mat, designated Rabbit Creek Spouter mat, formed a mound approximately 1 m×0.5 m of varying thickness up to several centimeters (Fig. 1A).

Internal mat temperatures ranged over 30–50°C because prevailing winds frequently blew the geyser spray away from the mat, allowing it to cool. The internal pH was about 8.0–8.2. The mat was fairly translucent and was composed of several colored layers of photosynthetic bacteria (Fig. 1B, C; see also Castenholz 1984). We sampled it for over a decade from 1981 until its demise in 1997, when the source dried up. The appearance of the layers varied over this time, probably in response to changes in summer weather conditions and temperature (Fig. 1B, C). In some seasons (Castenholz 1984), the surface was covered with a yellow-gold layer of *Phormidium* (*Leptolyngbya*) sp. (Fig. 1B). Deeper in the mat the layer of *Phormidium* sp. was green. At other times, the gold layer above the green layer was absent (Fig. 1C). A flesh- or olive-colored layer of *Chloroflexus* spp was sometimes found beneath the layer of *Phormidium* sp. (Fig. 1B). Deeper in the mat was a pronounced red to dark orange layer of filamentous bacteria (Fig. 1B, C). The filamentous bacteria of this red layer have been designated Rabbit Creek Red (RCR). The green layer of *Phormidium* sp. and the red filamentous layer were usually conspicuous (Fig. 1B, C). The layer of *Chloroflexus* spp was not always as conspicuous and varied more in its vertical distribution (Fig. 1B, C).

The translucence of the mat permitted deep penetration of light. Its highly layered nature resulted in a selective transmission of different parts of the visible and near-IR solar spectrum (Fig. 2A). A plot of the relative spectral irradiance with depth (Fig. 2) revealed the selective attenuation of radiation by pigments in the phototrophic layers described above. The strong attenuation over 400–500 nm at a depth of 0.5 mm was due to carotenoids and the Soret band of chlorophyll (Chl) *a* in the cyanobacterial surface layer. The absorption of light at 670 nm by Chl *a* and at 620 nm by phycocyanin in the cyanobacteria was clearly evident at a depth of 0.5 mm in the mat (Fig. 2B). At a depth of 1.5 mm, nearly all the radiation at 670 nm was attenuated, which would severely limit growth of cyanobacteria at any depth greater than this. Radiation was still available in the near IR, which could sustain the growth of *Chloroflexus* species and the red filaments.

In the red layer, the strong attenuation near 800 nm and 910 nm at depths greater than 3 mm (Fig. 2B) was attributed to Bchl *a*. Attenuation intensified with depth, causing prominent troughs in the spectra measured below 5 mm from the surface (Fig. 2C). The irradiance at 910 nm decreased to 30% of its solar surface value at a depth of 1.0 mm, to 10.4% at a depth of 5 mm, and to 0.59% at a depth of 10 mm. The irradiance at 1050 nm (a wavelength not absorbed by any of the pigments in the mat) likewise decreased to 33% of its surface value at a depth of 1 mm. However, in the region occupied by the red filaments, the irradiance at 1050 nm fell from its value of 17% at 5 mm to only 6%. The decrease in transmittance at 910 nm in that region was ten times greater than at 1050 nm. At the greatest depth shown, 13.5 mm (Fig. 2C), there was still radiation available at 970–1100 nm that could sustain phototrophic activity, but no organisms with a pigment absorbing in this region (i.e. Bchl *b*) were observed at this depth.

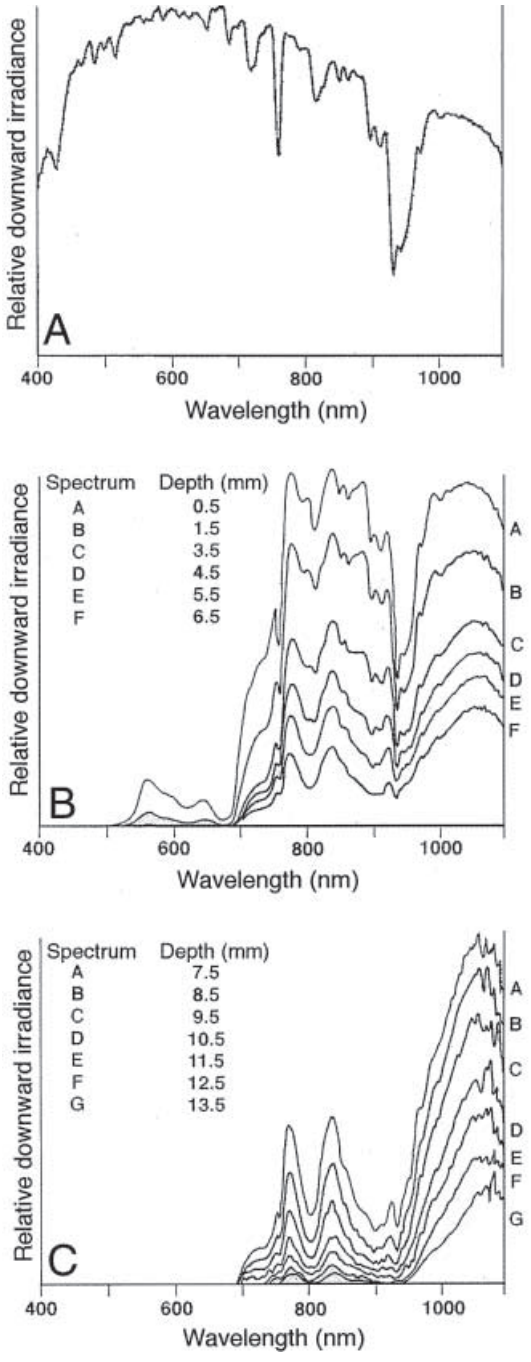

Fig. 2A–C Relative downward spectral irradiance with depth in Rabbit Creek Spouter mat measured with a spectroradiometer and fiber optic tip. **A** Spectral irradiance of the incident solar radiation at the surface of the mat. **B** Spectra at depths of 0.5–6.5 mm. **C** Spectra at 7.5–13.5 mm

The spectral irradiance depth profile (Fig. 2B, C) showed a spectral signature in the red and near IR unique to this community and distinct from that of other microbial mats described previously (Pierson et al. 1990, 1994; Pierson 1994; Castenholz and Pierson 1995). As in spec-

tral signatures of other mats (Pierson et al. 1990, 1994; Castenholz and Pierson 1995), the visible short wavelength part of the spectrum (400–700 nm) was strongly attenuated near the surface, while near-IR radiation penetrated to the greatest depth.

Morphology and ultrastructure

The RCR filaments from the deep red layer were indeterminate in length and about 1.5 μm in diameter (Fig. 3). Individual cells were 7–8 μm in length. Bright spots in the filaments as seen in phase contrast microscopy were probably poly-β-hydroxybutyrate (PHB) granules or other polyalkanoates. When massed, the filaments were a deep orange-red color. They showed no visible red fluorescence but fluoresced strongly in the near IR (data not shown). They stained gram-negative.

In transmission electron micrographs of thin sections (Fig. 4), the cells were packed with highly layered membranes very similar in appearance to those described in the purple filaments observed to inhabit the sheathed bundles of *Microcoleus chthonoplastes* (D'Amelio et al. 1987) and the unicellular purple sulfur bacterium *Tca. pfennigii* (Drews and Golecki 1995). No chlorosomes typical of green sulfur bacteria or *Chloroflexus* spp (Oelze and Golecki 1995) were observed. The RCR organisms were collected in situ from a low-intensity near-IR radiation field and may have produced a high cytoplasmic density of membranes to enhance their light-harvesting efficiency as seen in other phototrophs (Drews and Golecki 1995). Inclusions seen in the electron micrographs appeared similar to PHB. No other distinctive structures were observed.

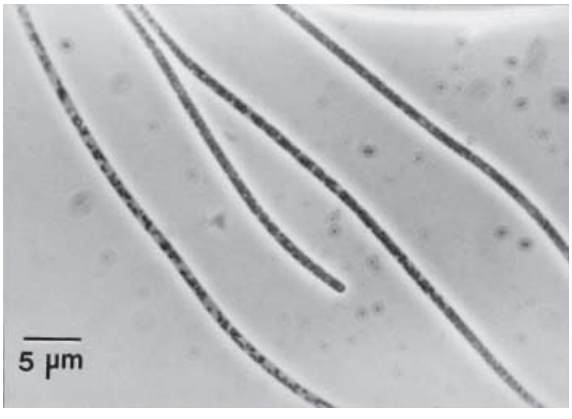

Fig. 3 Oil immersion photomicrograph of Rabbit Creek Red filaments. The *bar* represents 5 μm. Filaments are 1.0–1.5 μm in diameter. Each cell is approximately 8 μm long. *Dark inclusions* are probably poly-β-hydroxybutyrate (PHB) granules

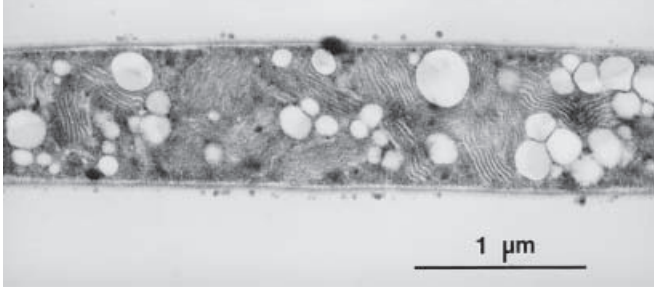

Fig. 4 Transmission electron micrograph of thin-sectioned Rabbit Creek Red (RCR) filament, using glutaraldehyde and osmium tetroxide fixation. Septate filaments contained PHB granules and patches of densely stacked membranes

Pigments

Extracts of the red mat layer in methanol had a large absorption maximum at 770 nm, as expected for Bchl *a* (Fig. 5). A small peak at 665 nm was occasionally seen and was probably due to Chl *a* in a few cyanobacteria contaminating this layer. Carotenoid and Soret Bchl *a* peaks were at 471 nm and 367 nm, respectively. The specific pigment content of Bchl *a* in the red layer was 5–6 μg (mg protein)$^{-1}$. Higher values of 13 μg (mg protein)$^{-1}$ were previously reported by Castenholz (1984). The specific pigment content of Bchl *a* within the red layer was similar to that of Chl *a* within the green cyanobacterial layer [4.0 μg (mg protein)$^{-1}$].

The in vivo spectrum of cells (Fig. 5) had absorption maxima at 913, 807, 674, 472, and 377 nm and was not identical to that of any other phototroph. The peaks at 913, 807, and 377 nm were probably due to the Bchl *a*. The long wavelength maximum was most similar to the spectra for Bchl *a* in vivo in *Chromatium tepidum* (Garcia et al. 1986) and the recently described *Roseospirillum*

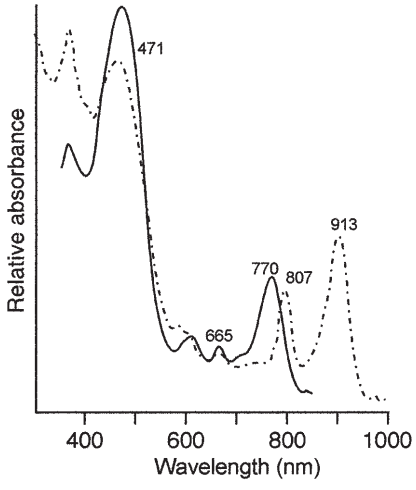

Fig. 5 Absorption spectrum of methanol extract of RCR filaments, showing typical peak for bacteriochlorophyll (Bchl) *a* at 770 nm (*solid line*) and in vivo absorption spectrum of RCR filaments (*dashed line*). Cells were sonicated in TSM buffer. The peaks at 913 nm and 807 nm correspond to Bchl *a*. The small peaks at 665 nm in methanol and 674 nm in vivo correspond to chlorophyll *a*. The peaks at 471–472 nm and 377 nm are composites of the carotenoid and Bchl *a* Soret peaks

parvum (Glaeser and Overmann 1999), both of which are non-filamentous purple bacteria. The peak at 674 nm was probably due to a small amount of Chl *a* from cyanobacteria. There was no evidence for Bchl *c* in any of the extracts, which is consistent with the absence of chlorosomes from the filaments. The carotenoids had their maximum absorption at 472 nm. With the Bchl *a* maxima of 807 nm and 910–913 nm, the filaments were well adapted for growth at the base of this thick mat of layered phototrophs, where only near-IR radiation is available (Fig. 1B, C, Fig 2).

Photosynthetic activity

Photoheterotrophy vs autotrophy

Suspensions of RCR were incubated with ^{14}C-labeled substrates to determine whether the cells were primarily photoheterotrophic or autotrophic. The filaments were incubated with bicarbonate or acetate, either in the light or dark, with or without sulfide as a potential electron donor or general reducing agent (Fig. 6). No evidence for light-dependent autotrophy was found and sulfide had no significant effect on the uptake of either substrate in the light or the dark. A strong light-dependent uptake of acetate occurred, indicating photoheterotrophy. This activity is typical of that of both *Htr. oregonensis* (Pierson et al. 1984) and *Cfl. aurantiacus* (Pierson and Castenholz 1995) growing in non-sulfidic hot springs. In situ, the RCR filaments probably obtain organic substrates from the autotrophic activity of the cyanobacterial layers found above them, as described for *Chloroflexus* spp in other hot spring mats (Ward et al. 1987).

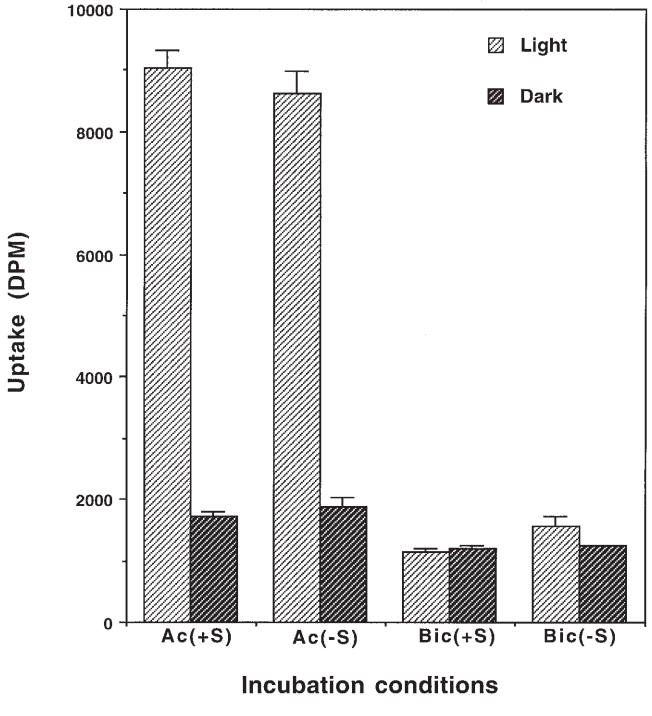

Fig. 6 Uptake of [^{14}C]-labeled acetate (*Ac*) and bicarbonate (*Bic*) by RCR filaments in the light or dark, with or without sulfide (*S*). Data are reported as the means of triplicate samples with standard deviations

Effects of light

The uptake of [^{14}C]-acetate by the RCR filaments incubated in full spectrum solar radiation was inhibited by high-intensity light (Table 1). Uptake was maximal over the range 150–600 W m^{-2}. The average level of uptake in low-intensity (300 W m^{-2} or less) and purely near-IR radiation was comparable to, if not higher than, those in the full spectrum incubations (Table 1). In a related experiment, the average uptake values were 8250–9050 disintegrations/min (DPM) over a range of near-IR intensities at 10–100% of solar IR. The maximum uptake in full spectrum solar radiation over the same range of intensities was 7000 DPM. These experiments suggest that, while the near-UV and visible parts of the spectrum may be inhibitory to phototrophy in RCR filaments, maximum activity occurs in intensities of near-IR radiation equivalent to the highest intensities of solar near-IR available.

Because the total irradiance within the mat environment was relatively low and limited to near-IR wavelengths (Fig. 2), we tested the RCR filaments for light-dependent uptake in situ by embedding uptake vials between the green and red layers in order to receive the same light field normally impinging upon the red layer (spectrum B in Fig. 2B). These vials had measurable light-dependent activity (19,789±1261 DPM) that was 135% of the dark control (14,709±250 DPM). The activity of the samples imbedded below the red layer (spectrum D in Fig. 2C) was not significantly above that of the dark control (15,360±188 DPM, which was 104% of the dark control). Samples incubated in the full intensity and full spectrum light field above the green layer (Spectrum in Fig. 2A) were so strongly photoinhibited that levels of uptake were depressed substantially (3603±146 DPM) to 24% of the dark controls. The filaments became bleached and all

Table 1 [^{14}C]-Acetate uptake as a function of light intensity and near-infra-red (*Near IR*) radiation. Each disintegrations/min (DPM) value is mean ± standard deviation (n=3) and all values were adjusted by subtracting uptake in the dark (1150 DPM)

Light intensity (W m^{-2})	DPM
Visible	
900	5703.3± 930.9
750	6956.7± 166.3
600	7883.3± 353.1
300	7530.0± 502.6
150	7556.7± 800.0
Near IR	
300	8083.3± 296.3
260	7503.3±1173.7
210	7920.0± 580.5
120	8356.7± 196.2

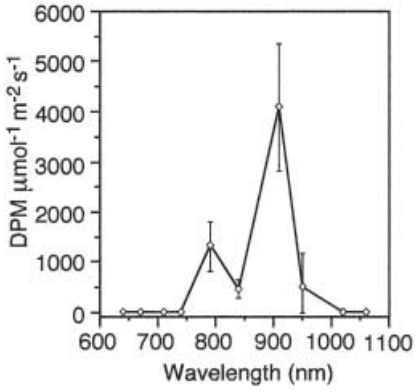

Fig. 7 Uptake of [^{14}C]-acetate as a function of incubation in different wavelengths of visible and near-IR radiation. Specific wavelengths were defined using narrow-band interference filters: 640, 670, 710, 740, 790, 840, 910, 950, 1020, and 1060 nm. Uptake under each lighting condition was corrected for the dark control. Data are reported as means of triplicate samples with standard deviations

motility was destroyed. Thus, the RCR filaments are adapted to the relatively low-intensity near-IR light environment in which they grow in these mats.

The action spectrum of the red filaments (Fig. 7) confirms that the photosynthetic activity was maximal near 800 nm (790 nm) and 910 nm, consistent with the in vivo red and near-IR absorption spectrum of the red layer filaments (Fig. 5) and the spectral irradiance measured beneath the red layer deep in the mat (Fig. 2). These observations, coupled with the in situ uptake experiment and the near-IR vs full spectrum uptake data discussed previously (Table 1), suggest that these bacteriochlorophyll absorption maxima are the major wavelengths used to sustain photosynthesis in the RCR filaments.

Effects of temperature and oxygen

Photosynthetic activity was greatly reduced below 30 °C and above 50 °C (Fig. 8). The optimum was 34–46 °C (Fig. 8), consistent with the observed range of temperatures measured within the mat. The temperature range for phototrophic activity seen here could reflect an adaptation of the entire population to the range of temperatures found within this mat, or it could reflect the presence of different temperature-adapted strains with maximal activity within specific, more limited temperature ranges.

Oxygen profiles

Microelectrode measurements of oxygen within mat exposed to high-intensity light showed that the zone of maximum oxygen concentration was in the top 3 mm (Fig. 9). The maximum rate of oxygen production (93 µmol l^{-1} min^{-1}) occurred at 1.5–2.0 mm. Oxygen levels fell sharply within the red layer under all conditions in three different profiles, supporting the conclusion that the red filaments are anoxygenic phototrophs. Preliminary experiments suggested that RCR filaments incorporated similar levels of acetate in anoxic and in oxic conditions (data not shown). The RCR filaments apparently can tolerate exposure to oxygen produced in the cyanobacterial layers, since substantial oxygen diffuses to the depth of the red layer under some conditions (Fig. 9).

Motility

The RCR filaments showed gliding motility on an agar surface and exhibited a distinctive "puffing behavior" when excised sections of the mat were incubated in the dark or low light. Dark orange "puffs" of filaments ap-

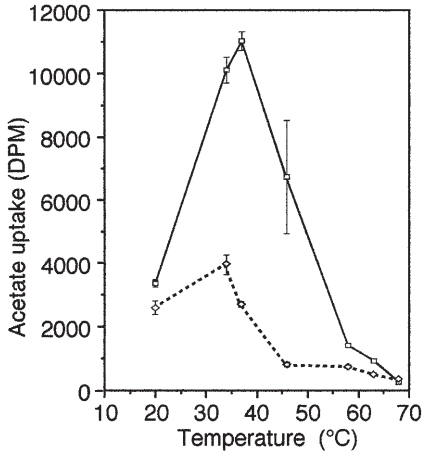

Fig. 8 Uptake of [^{14}C]-acetate as a function of incubation in different temperatures. RCR filaments were homogenized in spring water and incubated with [^{14}C]-acetate in the light (*solid line*) or in the dark (*dashed line*) for 1 h. Data are reported as means of triplicate samples with standard deviations

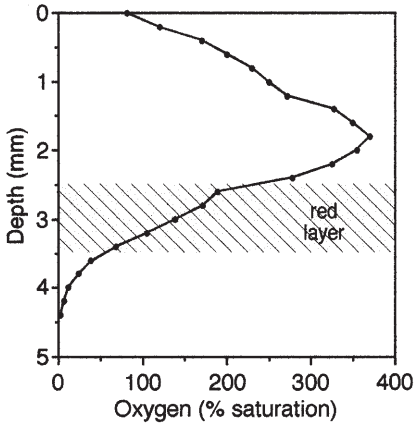

Fig. 9 Oxygen depth profile of Rabbit Creek Spouter mat. Depth profile of oxygen concentration was measured with a microelectrode under high-intensity light (800–850 W m^{-2}) and at 40–43 °C. The cyanobacterial layer reached a depth of about 2.5 mm; and the red layer of filaments extended from 2.5 mm to about 3.5 mm. An olive-colored layer was found below this

peared on the cut surfaces (Fig. 1D). These puffs were examined microscopically, with electron microscopy, and with absorption spectroscopy; and they appeared nearly identical to the red layer RCR filaments from within the mat. The near-IR absorption maxima of the puffs were at 806 nm and 905 nm.

Distribution of red/orange filaments at other thermal sites in Yellowstone

We examined several other mats in Yellowstone for the presence of deep orange or red layers with Bchl-*a*-containing filaments of similar diameter, and found several sites with similar characteristics. There is considerable variation in the position of the two in vivo IR absorption maxima in undermat samples. The red undermats that were most similar to RCR filaments were found at Fairy Geyser (807 nm and 912 nm) and Octopus Spring (805 nm and 908 nm). Absorption maxima of undermats at Painted Pool (796 nm and 867 nm) and Imperial Geyser (795 nm and 877 nm) were more similar to those of *Htr. oregonensis* (800 nm and 865 nm; Pierson et al. 1984). Mats at Spray Geyser (799 nm and 893 nm) and Hillside Springs (799 nm and 889 nm) had near-IR peaks at longer wavelengths than *Htr. oregonensis*, but not greater than 900 nm.

Of particular interest was the presence of RCR absorption characteristics in the orange undermat of the well studied Octopus Spring (Fig. 1E, Fig. 10). Previously this undermat layer had been assumed to be primarily *Cfl. aurantiacus* (Doemel and Brock 1977; Brock 1978), although their studies did not include in vivo absorption spectroscopy. Molecular analyses revealed much greater diversity (Ward et al. 1998). In an absorption spectrum of the entire undermat (data not shown), we detected pigments typical of both *Cfl. aurantiacus*, as indicated by the absorption maximum at 745 nm, and RCR-like filaments, as indicated by peaks at 799 nm and 905 nm. When the surface of the mat was darkened for 24 h, the green cyanobacterial layer became covered with bright orange puffs, due to the migration of filamentous bacteria up through the cyanobacterial layer (Fig. 1F). Absorption spectra of collected puffs had peaks at 909 nm and 799 nm, but none at 745 nm, suggesting that they were RCR-like filaments, not *Chloroflexus* spp.

We sectioned the Octopus Spring mat into specific colored layers to determine the precise locations of *Chloroflexus* spp and RCR-like filaments using in vivo absorption spectroscopy. The surface green layer of *Synechococcus* sp. (see Fig. 1E) had prominent peaks for Chl *a* (677 nm) and Bchl *c* (738 nm) with broad absorbance in the near-IR, consistent with *Chloroflexus* spp (Fig. 10A). The second layer down was dark orange and had some Bchl *c* (745 nm) and prominent peaks at 806 nm and 909 nm, typical of RCR filaments (Fig. 10B). The third layer down was olive colored and had a prominent Bchl *c* peak at 744 nm, again consistent with *Chloroflexus* spp (Fig. 10C). The bottom orange layer appeared to be a mix of many different chlorophylls (peaks at 667, 743, 799, and 879 nm), and may represent older accumulated mat layers (Fig. 10D).

These results demonstrated the presence of several novel populations. First, there appeared to be at least two different populations of Bchl-*c*-containing *Chloroflexus*-like organisms, one at the surface (738 nm) and one beneath the surface (744–745 nm) that became more prominent deeper in the mat. Second, the layer just beneath the surface contained RCR-type filaments. These data corroborate immunolabeling and molecular analyses which suggest that the phototrophic populations of hot spring communities are more diverse than previously thought (Tayne et al. 1987; Ward et al. 1998). The distribution of these phototrophs, however, may not be consistent year to year.

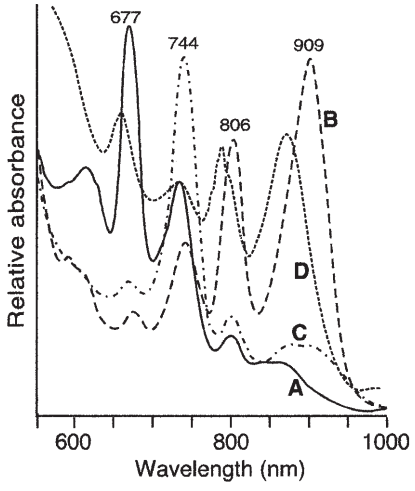

Fig. 10A–D In vivo absorption spectra of selected layers of Octopus Spring mat. **A** Spectrum of surface cyanobacterial layer. **B** Spectrum of orange/red layer immediately beneath cyanobacterial layer. **C** Spectrum of deeper olive-colored layer. **D** Spectrum of bottom orange-colored layer. See text for identification of absorption maxima

Comparison with Htr. oregonensis

The diameter, active motility, photoheterotrophy, and the presence of only Bchl *a* in the RCR filaments were characteristics similar to those of *Htr. oregonensis* (Pierson et al. 1984, 1985). The ultrastructure, in vivo absorption maxima, and sensitivity to high-intensity light of RCR filaments were characteristics that differed from those of *Htr. oregonensis*. We analyzed the mats at Kahneeta Hot Springs in Oregon to see whether we could detect evidence for RCR-type filaments. The absorption maxima for the bacteriochlorophylls in typical *Htr. oregonensis* were at 795 nm and 865 nm (Pierson et al. 1984, 1985). Those spectra were prepared from selected loose tufts of gliding filaments that formed the mat surface. Spectra of the whole undermat (data not shown) revealed peaks at 903 nm and 804 nm, similar to Bchl *a* in RCR filaments, 743 nm attributed to Bchl *c* in *Cfl. aurantiacus*, and

678 nm attributed to Chl *a* in cyanobacteria. This spectrum was similar to the undermat spectrum at Octopus Spring. When the mat of *Htr. oregonensis* at Kahneeta was covered in situ or when excised mat samples were covered for 3 days, the surface became covered with dark red filaments resembling the red filaments at the base of the Octopus Spring mat and the Rabbit Creek red layer. The spectrum of the red layers that accumulated in the dark on the surface of Kahneeta mat samples had peaks at 913, 803, 482, and 376 nm, very similar to those of the orange/red filaments at Rabbit Creek and Octopus Springs.

The filamentous bacteria at Kahneeta Hot Springs are especially enigmatic. The filaments covering the surface of the mats under undisturbed conditions (*Htr. oregonensis*; Pierson et al. 1984, 1985) have neither the same absorption maxima nor the same ultrastructure as the red filaments that appear only when the mat is darkened. Orange surface filaments collected and incubated in the spring water in flasks open to the air but kept dark did not turn red. The absorption maxima of whole undermat revealed the peaks associated with the red filaments, suggesting that there are two different populations of Bchl-*a*-containing filaments at Kahneeta: the red filaments dwelling deep in the mat and the orange surface-dwelling *Htr. oregonensis*. As with the Octopus Spring mat, darkening the mat caused the deeper filaments to migrate to the surface.

General conclusions

Our survey of Yellowstone hot springs has shown that Bchl-containing organisms are abundant, forming conspicuous mat layers at temperatures of 37–56 °C. In most springs in Yellowstone, these organisms form lower layers in the mats below layers of cyanobacteria and sometimes beneath a layer of *Chloroflexus* spp. Some have distinctive near-IR absorption maxima greater than 900 nm; and those we examined with electron microscopy have novel arrangements of intracellular membranes. Their migration is also distinctive, although the cues are unknown. It is interesting to note that recent research applying molecular techniques to the mats at Octopus Spring revealed the presence of 16S rRNA sequences that are related to but not identical with those of *Cfl. aurantiacus* and *Htr. oregonensis* (Weller et al. 1992; Ward et al. 1998). The 5S (Pierson et al. 1985) and partial 16S (Ward et al. 1998) rRNA sequences of *Htr. oregonensis* are different from but clearly related to those of *Chloroflexus* spp. It thus appears that there are other filamentous phototrophs containing Bchl *a* in hot springs that remain to be identified.

Although most of the evidence suggests otherwise, it is possible that the red filaments are genetically identical to *Htr. oregonensis* and that expression differences account for varying membrane content and a shift in Bchl *a* absorption maxima. In other phototrophs, such phenotypic variation is regulated by environmental conditions such as light intensity and oxygen level (Drews and Golecki 1995). Isolation in culture and/or the application of molecular techniques will, however, be necessary to determine the precise relationship, if any, among these filamentous phototrophs. Attempts to culture *Htr. oregonensis* and the various orange/red filaments in our laboratories have been relatively unsuccessful. Thus, we are currently pursuing molecular approaches to identify novel Bchl-*a*-containing filaments observed in hot spring communities, including those sites described here in Yellowstone National Park and Kahneeta Warm Springs.

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