Cyanobacteria in Geothermal Habitats

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Summary

In the last decade advances in high-throughput DNA and RNA sequencing have driven more intensive surveys of cyanobacterial diversity in geothermal systems worldwide and the development of a deeper understanding of well-studied hot spring cyanobacterial communities. As a consequence, it is now possible to build, atop the long-term studies of these systems based on morphological, pure-culture and initial 16S rRNA observations, a more thorough understanding of the biogeographical and local distributions of cyanobacteria in these settings. Population genetics studies with increased molecular, spatial and temporal resolution have begun to define the ecological species populations of thermophilic cyanobacteria and to reveal the processes that drove their evolution and current ecology. Metagenomic studies have begun to reveal the functional gene repertoire of the predominant cyanobacteria and associated members of communities in which they reside and with whom they interact. Gene expression studies, including metatranscritomic studies, have begun to reveal patterns of *in situ* gene expression.

3.1 Introduction

Dramatic advances in high-throughput molecular approaches in microbial ecology have, in turn, dramatically improved understanding of thermophilic cyanobacteria and the communities they inhabit. The chapter contributed to the

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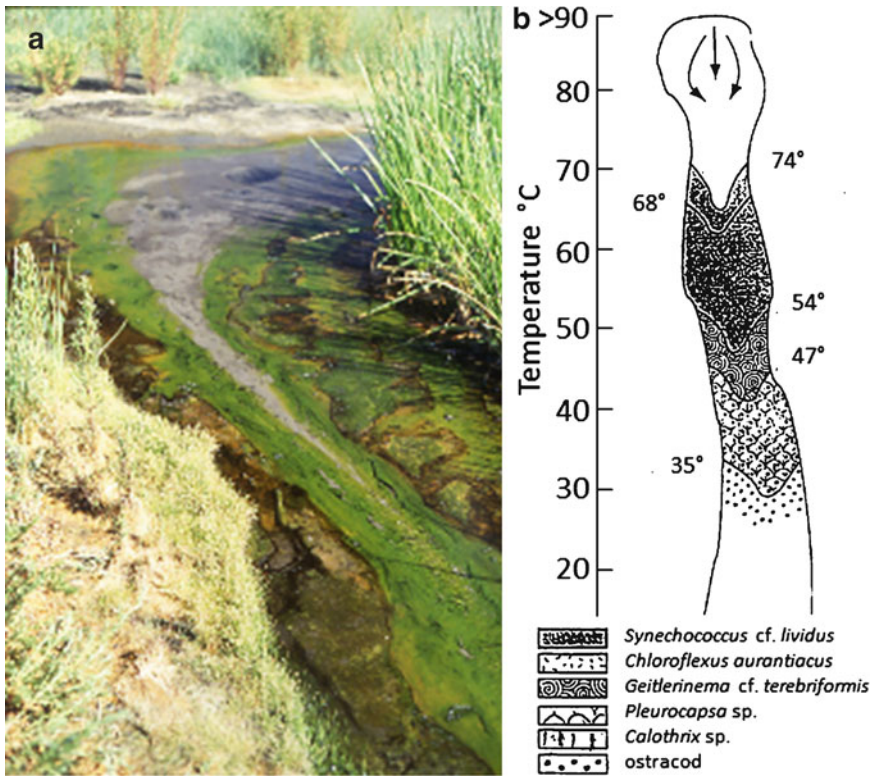


Fig. 3.1 Hunter's Hot Springs, Oregon: (**a**) Photograph and (**b**) schematic depicting the approximate downstream distribution of the predominant cyanobacteria, *Chloroflexus aurantiacus* and one grazing invertebrate present at the mat surface in the outflow of most springs in the Hunter's Hot Spring group

previous edition of this book (Ward and Castenholz 2000) emphasized how molecular methods developed in the 1990s had reshaped our knowledge of cyanobacteria in geothermal habitats. In that chapter, we compared the traditional view offered by microscopy and cultivation with the view emerging through 16S rRNA analyses. We also expanded the description of well-studied geothermal microbial communities initiated by Castenholz (1973a) with the description of Hunter's Hot Springs, OR (Fig. 3.1) by adding a description of work conducted at Octopus Spring in Yellowstone National Park (YNP). This new chapter emphasizes what has been learned in the past decade as 16S rRNA methods have been used to more extensively survey the cyanobacteria of geothermal habitats and, as genomics, metagenonomics, metatranscriptomics and metaproteomics have deepened understanding of cyanobacteria in well-studied model systems in Mushroom Spring (Fig. 3.2) and White Creek (Fig. 3.3) in YNP. As a result, we rely on the previous chapter (Ward and Castenholz 2000) for coverage of work completed prior to 2000. Many portions of that chapter have been omitted and reference will be made to figures and tables of that chapter when necessary. The cyanobacteria found in geothermal habitats, based on both phenotype and genotype are listed in Table 3.1.

3.2 Distribution of Thermophilic Cyanobacteria Based on Morphology and Cultivation

Differences in the composition of cyanobacterial populations among diverse hot springs can often be discerned by microscopic examination, based on distinctive morphological characteristics (Table 3.1 and Fig. 3.2; see also Fig. 1 of Ward and Castenholz (2000)), although true genotypic differences or similarities are not revealed by microscopy. Differences observed between springs a few meters or kilometers from each other are likely to be due to local variations in chemical composition, temperature or exposure to solar irradiance. Those differences observed among widely separated springs are more likely the consequence of geographic isolation and the limitations of dissemination. Morphology can also be assessed from culture isolates from the springs.

3.2.1 Geographic Distribution

Geothermal springs are located non-continuously and may be likened to islands. They are scattered on all continents except Antarctica (but steam vents occur on two Antarctic

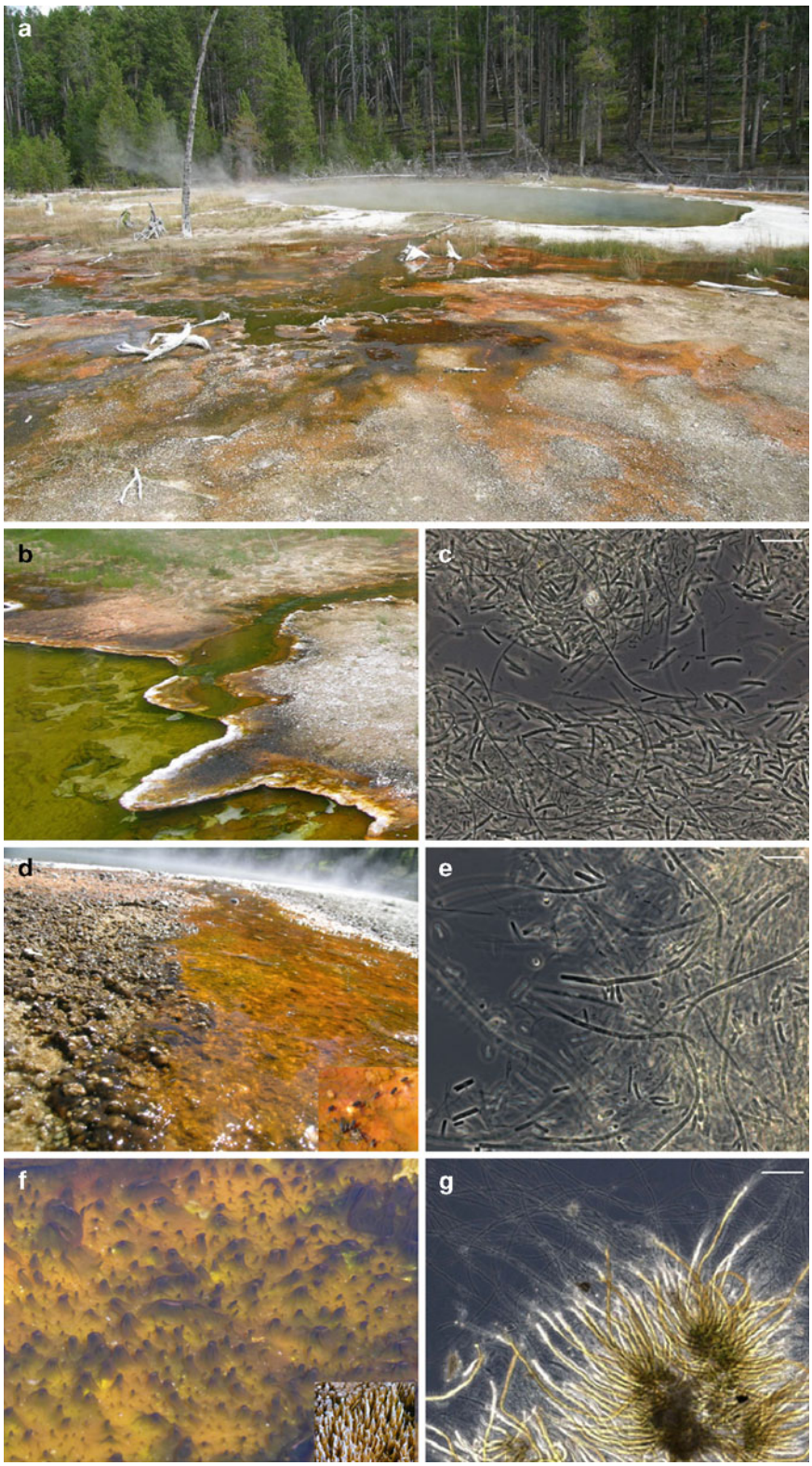


Fig. 3.2 Mushroom Spring, YNP and its microbial communities: (**a**) Landscape showing the *yellow-green mat* lining the source pool and downstream mats in foreground; (**b**) *Synechococcus* mats in source pool and effluent channel; (**c**) Photomicrograph of *Synechococcus* cells and filaments in *upper green mat* layer; (**d**) *Low-angle view* of downstream *Leptolyngbya* (*Phormidium*) (*orange mat* on *right*) and *Calothrix* (*brown tufts* on *left*) communities with source pool in background at *top* of photo; *inset* shows ephydrid flies on *Leptolyngbya* (*Phormidium*) mat; (**e**) Photomicrograph of *Leptolyngbya* (*Phormidium*) filaments together with *Synechococcus* cells and bacterial filaments from an *orange mat* like that shown in (**d**); (**f**) Conical structures in a *Leptolyngbya* (*Phormidium*) mat within a quiescent pool; *inset* shows silicified cones upon dehydration; (**g**) Photomicrograph of *Calothrix* filaments typical of those in the *brown mat* in (**d**). All photomicrographs are phase contrast images; scale bar is ~10 μm in (**c** and **e**) and ~100 μm in (**g**)

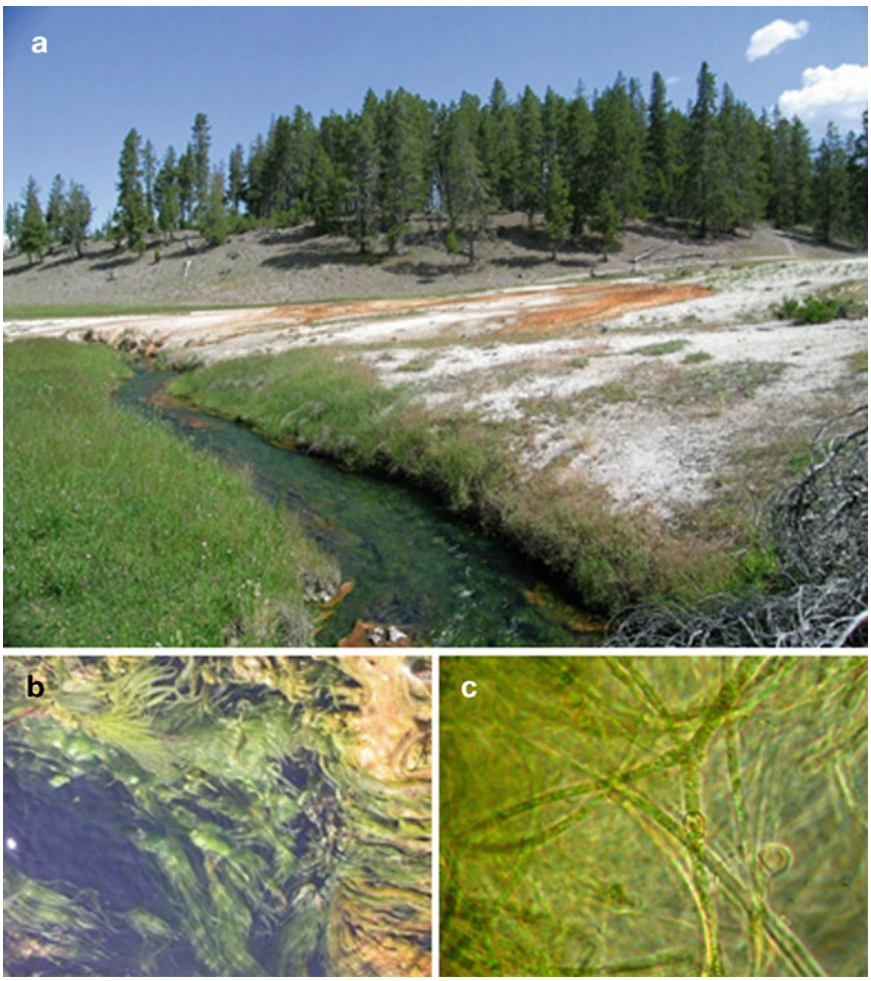


Fig. 3.3 White Creek, YNP: (**a**) *Landscape view* looking downstream with lower temperature influents from Octopus Spring and other nearby hot springs to the *right*; (**b**) Streamers containing *Fischerella laminsosus* are found below ~55°C; (**c**) Phase contrast micrograph of White Creek mat (~53°C). Note the true-branching pattern and heterocyst of *F. laminosus* near image *centre*

volcanoes) and on many island groups. Hot springs are mainly associated with current or recent volcanic activity, but also with active faulting where surface waters sink deeper, are heated and then resurface. Often there are great distances separating hot spring clusters. It is not unreasonable to believe that there should be endemic species of thermophiles, restricted to certain hot spring groups as a result of geographic isolation and evolutionary divergence of the microbes. This concept should not be surprising, since dispersal of obligate thermophiles from rare and distant point sources is certainly a limiting factor for some taxa, and the time between successful long-distance disseminations may be enough to allow speciation to take place.

A striking anomaly suggesting geographically restricted distributions of thermophilic cyanobacteria is that all forms of thermophilic *Synechococcus* appear to be absent from Icelandic hot springs (although numerous springs exist there that appear chemically suitable) (Castenholz 1978) (Table 3.1). Thermophilic *Synechococcus* (with slightly different morphologies) occur in New Zealand and European springs, but include only forms that grow up to temperature limits of about 62°C and 58°C, respectively (Castenholz 1969, 1976, 1978, 1996; Ward and Castenholz 2000). Forms of *Synechococcus* that grow in nature up to limits of 73–74°C in the western contiguous United States and south into Central and South America, are absent in the geographic regions mentioned above, although they extend into eastern Asia (Thailand (Sompong et al. 2008; Castenholz, unpublished data) and China (Yun 1986)). In contrast, some thermophilic cyanobacteria, such as *Fischerella (Mastigocladus) laminosus* morphotypes and the high temperature forms (HTF) of "*Chlorogloeopsis*" appear to be cosmopolitan in distribution (Castenholz 1996). Latitude and daylength do not appear to be very important in the distribution of thermophilic cyanobacteria, since many morphotypes similar to those of lower latitude springs occur in the hot springs of the east coast of Greenland at 70–71°N. These springs experience complete darkness in winter and constant daylight in summer (Roeselers et al. 2007).

Table 3.1 Cyanobacteria found in geothermal habitats, arranged top to bottom in order of their occurrence along a thermal gradient, their genetic affiliation, representative cultivars and distribution relative to biogeography and environmental parameters

Morphological genus species	16S rRNA genotype	Representative strains	Biogeographical distribution based on morphotype (M), environmental sequence (E) or sequenced laboratory strain (L)															Max °C	pH	Sulfide tolerance	N_2 fixation
			NA	LA	NZ	JA	PH	TH	TI	IC	EU	AU	AF	GR	RU	CH	ME				
Synechococcus cf. *lividus* (HTF)			M	M				M								M		73–74	>6	Low	
	A″		E															72			Yes
	A′		E															72			Yes
		Miller Group IV	L																		
	A		E,L															67			
		Miller Group III	L																		
		Allewalt JA-3-3Ab																			Yes
Synechococcus cf. *lividus*			M	M	M	M	M	M	M		M		M			M		58–66	>pH 5	Low (varies)	Varies
	B′	Allewalt JA-2-3B′a(2-13)	E,L															63			Yes
		Miller Group II	L																		
	B	Allewalt TS-15	E,L															57			
	T1-like		E	E				E,L	E						L		E				
	C1-like		E,L			E,L	E	E,L	E								E				
	C9-like		E,L		E	E		E									E				
Cyanothece (*Synechococcus*) cf. *minervae*			M,E,L	M					E									62	>6	Low (?)	No
"*Chlorogloeopsis* HTF"			M	M	M					M	M							64	>4.5	Low (~0.15 mM)	Yes
Leptolyngbya (*Phormidium*) spp. (cf. *P. laminosum*)			E,L				E				L						E	~62		Mod. (?)	No
	OS Type I		E																		
Fischerella (*Mastigocladus*) cf. *laminosus*			M, E,L	L	L	E,L	E	E		L	L	E					E,L	58	>5	Mod. (~0.25 mM)	Yes
Leptolyngbya (*Oscillatoria*) cf. *amphigranulata*					M, E,L	E	E	E				E		L	L			~56	>6.5	High (~3 mM)	No
Geitlerinema (*Oscillatoria*) cf. *terebriformis*			M,L	M												M	L	55	>6	High (~1 mM)	No
Spirulina cf. *labyrinthiformis*			M															51	>6	Mod (~0.1 mM)	No
Calothrix spp.			M															~53-55	8-8.5	Low (?)	Yes
Pleurocapsa spp.			M															57			Yes

The first three columns are hierarchical in the sense that each morphotype is associated with genotypes in following rows and each genotype is associated with strains in following rows. For instance, the morphotype *Synechococcus* cf. *lividus* (HTF) is grouped with three A-like genotypes; one strain representing genotype A′ and two strains representing genotype A and have been cultivated

The genomes of strains JA-2-3Ab, a close relative of strain JA-3-3Ab (see Allelwalt et al. 2006), and JA-2-3B′a(2-13) have been obtained (Bhaya et al. 2007)

NA North America, *LA* Latin America, *NZ* New Zealand, *JA* Japan, *PH* Phillipines, *TH* Thailand, *TI* Tibet, *IC* Iceland, *EU* Europe, *AU* Australia, *AF* Africa, *GR* Greenland, *RU* Russia, *CH* China, *ME* Middle East

(?) unknown, but inferred from ecological setting

3.2.2 Distributions Determined by Temperature and Chemistry

Within a local biogeographical region, temperature and pH, in combination with availability of combined nitrogen, phosphorus and other nutrients and/or concentration of free sulphide (i.e. H_2S, HS^-, S^{2-}) determines the distribution of thermophilic cyanobacteria (Table 3.1).

3.2.2.1 Temperature

Table 3.1 is organized to show the approximate distributions of thermophilic cyanobacteria relative to temperature (high to low listed top to bottom). The most notable differences in a biogeographical sense are the limited distributions of high-temperature forms. Since all of the listed taxa are not present in a particular system, we will rely on Sect. 3.2.3 to provide further insight into distribution related to temperature in well-studied systems.

3.2.2.2 pH

In hot springs worldwide, cyanobacteria are not observed below a pH of about 4.0, and their diversity seems quite limited below pH 6 (Brock 1973). In Yellowstone (Clearwater Springs and Norris Geyser Basin) *Synechococcus* spp. or varieties occur in hot springs with pH values as low as ~5.2, and "HTF *Chlorogloeopsis*" populations occur at pH levels as low as ~4.5 (see Ward and Castenholz 2000). The *Synechococcus* (clone Y-7C-s) isolated from a pH 5.5 spring in the Clearwater group grew at maximal rates only at pH levels above pH 7 and thus appeared acidotolerant not acidophilic (Kallas and Castenholz 1982a, b).

3.2.2.3 Nitrogen and Phosphorus Availability

When the outflows of neutral to alkaline, non-sulphidic hot springs containing combined nitrogen have cooled to 73–74°C there is the likelihood (at least in the above mentioned geographic regions) that a high temperature form (HTF) of *Synechococcus* will be present as a biofilm or mat, which may, in turn, influence the chemistry downstream where other species of cyanobacteria enter the thermal gradient. For example, the combined nitrogen (usually as NH_4^+) in the spring source may be largely removed by *Synechococcus*. The *Synechococcus* ecotypes or species may then be succeeded downstream (at least below ~58°C) by heterocystous, N_2-fixing, cyanobacteria, most commonly *Fischerella (Mastigocladus) laminosus* (e.g. White Creek, Lower Geyser Basin, YNP) but elsewhere by *Calothrix* spp. below ~53–55°C (unpublished observations). Nitrogen fixation has been measured at approximately 60°C in hot spring *Synechococcus* (Steunou et al. 2006, 2008), and at lower temperatures where heterocystous *Fischerella* and *Calothrix* occur (Stewart 1970; Wickstrom 1980). In contrast, a spring may be rich enough in combined nitrogen to favour *Fisherella* without heterocysts (Miller et al. 2006). In other cases, combined nitrogen may be very low at the source, even in high-temperature springs, and heterocystous cyanobacteria may constitute the upper-temperature species at the upper limit for growth (e.g. "HTF *Chlorogloeopsis*" at 64°C in New Zealand). Possible distribution differences based on phosphorous availability have been suggested by comparison of genomes of *Synechococcus* strains representative of populations at different positions along the flow path (Sect. 3.4.1.1).

3.2.2.4 Sulphide Tolerance and Utilization

Many neutral to alkaline geothermal springs contain primary soluble sulphide in the source water. Sulphide is an effective inhibitor of photosynthesis and possibly other physiological processes in the majority of cyanobacteria, but may be used as a photosynthetic electron donor in some sulphide-tolerant species (Cohen et al. 1986; Castenholz and Utkilen 1984). Present evidence indicates that no thermophilic cyanobacteria with the capacity to grow above 56°C are capable of growing in waters with more than ~10 μM sulphide (Castenholz 1976, 1977; Garcia-Pichel and Castenholz 1990). In sulphide-rich springs of New Zealand the upper-temperature, sulphide-tolerant and sulphide-utilizing cyanobacterium is a *Leptolyngbya* ("*Oscillatoria*") *amphigranulata* morphotype (Castenholz 1976). In some hot springs of the upper Mammoth Terraces, Yellowstone Park, where source temperatures are ~52°C or below, a sulphide-utilizing *Spirulina labyrinthiformis* morphotype (with an upper temperature of 51–52°C) predominates near the sulphide-rich source (Castenholz 1977). In higher temperature springs, with similar sulphide concentrations at the source, waters usually lose all detectable sulphide by the point where the outflow reaches 52°C and are dominated by less sulphide-tolerant species at that temperature and below. Non-photosynthetic sulphide-oxidizing bacteria (e.g. *Sulfurihydrogenibium*) predominate in zones below 75–77°C sources in several sulfidic hot springs in the upper terraces of Mammoth Hot Springs, YNP (e.g. Inskeep et al. 2010) and are likely to be mainly responsible for sulphide removal in the upper temperature zone. Icelandic and Yellowstone springs with primary sulphide often have mats of photoautotrophic filamentous anoxygenic bacteria (FAPs; e.g. *Chloroflexus, Roseiflexus*) at temperatures of ~66°C down to a temperature where surface-water sulphide disappears (<~60°C) (Castenholz 1973b; Giovannoni et al. 1987; Klatt et al. 2012b). In a few Icelandic mats, sulphide-oxidizing FAPs on the mat surface remove sulphide, permitting sulphide-sensitive cyanobacteria to grow beneath them in the lower part of the photic zone (Jørgensen and Nelson 1988).

3.2.2.5 Salinity

Although saline hot springs are rare, those that arise as hot altered seawater on the Reykjanes peninsula of southwestern Iceland (mid-Atlantic Ridge terrestrial outflow)

harbor cyanobacteria at temperatures near 40°C. However, isolates of *Leptolyngbya* from this water (the Blue Lagoon) grew well at three times the salinity of seawater and at a temperature of 55°C, which was also an unexpected characteristic of some *Leptolyngbya* isolates from endolithic habitats in ancient Yellowstone travertine (Banerjee et al. 2009).

3.2.3 Well-Studied Mat Systems

Brief consideration of cyanobacterial diversity based only on morphology and cultivation is provided in this section. More detailed descriptions based on molecular sequence data are presented in Sects. 3.3 and 3.4.

3.2.3.1 Hunter's Hot Springs, Oregon

These privately owned springs, 3.5 km north of Lakeview, Oregon (elev. 1,470 m), have been studied periodically by Castenholz and students since the early 1960s (Fig. 3.1a). They consist of a series of springs initiating from an active deep faulting system, many issuing at temperatures of about 90°C with a pH of 8.0–8.4. The major ions are sodium (~8.6 mM), chloride (~3.2 mM), silicate (~1.2 mM), bicarbonate (~1.7 mM) and sulphate (~2.8 mM) (specific conductance of ~1.1 mS). The chemistry and microbiota of Hunter's Hot Springs is characteristic of many hot springs of the Great Basin (C.E.Wingard and R.W.Castenholz, unpublished data; Mariner et al. 1974).

Besides identifying the conspicuous cyanobacteria, the objective of early studies was to explain the abrupt upper and lower temperature boundaries of distinctive phototrophic populations in a continuous and linear temperature gradient. These distributions are illustrated in Fig. 3.1b and are described in Castenholz (1969, 1973a) and Wickstrom and Castenholz (1978, 1985). Briefly, the upper temperature limit for cyanobacteria, and for global photosynthesis, is almost certainly 73–74°C and this boundary is easily seen when the temperature remains relatively constant at a particular point in the outlet. This greenish-yellow mat, composed of a cyanobacterial form-species identifiable morphologically as *Synechococcus lividus* Copeland occurs as the top cover of the mat in most of the Hunter's Spring outflows to about 54–55°C where it is abruptly replaced by a dark reddish-brown cover of *Geitlerinema* (*Oscillatoria*) *terebriformis*, a distinctive form identified by morphology, physiology, ecology, and recently by 16S rDNA sequences (Castenholz 1978, 1996, and T.B. Norris, unpublished sequence data). The uniform green cover of *Synechococcus*, however, was found to consist of at least four stable thermotypes that appear microscopically similar (Peary and Castenholz 1964; Miller and Castenholz 2000) (Fig. 3.4a, c). In culture, the most thermotolerant clone grew at a maximum rate at 63–68°C, but grew up to a temperature of 72°C and not below 55°C (Meeks and Castenholz 1971; Miller and Castenholz 2000). In culture, with continuous illumination, the maximum growth rate of this strain was slightly <1 doubling/24 h, considerably slower than the lower temperature strains, which were able to grow optimally at temperatures well below 55°C, but which could not grow at the high temperature of the most thermotolerant strain (Miller and Castenholz 2000). The doubling rates in Peary and Castenholz (1964) were considerably greater than those in Miller and Castenholz (2000) (compare Fig. 3.4a, b), possibly due to differences culture conditions with higher light intensity in Peary and Castenholz (1964) or in the genotypes of the strains.

G. terebriformis has an upper growth temperature limit of 54.5°C in culture (and in nature) and grows at a maximal rate close to this upper limit (Castenholz 1968, 1973a). Since it is highly motile (by gliding), the upper edge of the mat adjusts its position to its upper temperature limit. Since the mat is generally thick enough to absorb over 95% of visible radiation, this cover sets the lower boundary of the otherwise extensive *Synechococcus* mat, which subsequently becomes light-limited. Although all the clonal cultures of *G. terebriformis* have shown growth rates of over 2 doublings/24 h below 48°C (Fig. 3.4a), in most springs of E. Oregon and N. Nevada, the *Geitlerinema* mat ends abruptly at about 47–48°. This is a result of dense, voracious populations of the thermophilic and "herbivorous" ostracod *Thermopsis thermophila* (ex *Potamocypris* sp.) that are nearly ubiquitously distributed in the same geographic region (Castenholz 1973a; Wickstrom and Castenholz 1985; Külköylüoglu et al. 2003).

Since this species of ostracod can survive and reproduce at temperatures as high as 48°C and possibly higher (Wickstrom and Castenholz 1973; Külköylüoglu et al. 2003), it ingests the delicate trichomes of *Oscillatoria* and the soft undermat of *Chloroflexus* at a rapid rate. However, the cyanobacterial population below this temperature is composed primarily of the highly grazer-resistant *Pleurocapsa* sp. and *Calothrix* sp. The tapered filaments of *Calothrix* are embedded within the nearly amorphous mass of *Pleurocapsa* cells (Wickstrom and Castenholz 1978). The ostracods appear to graze primarily on the exposed lawn of the tips of *Calothrix* filaments which continue to grow between the *Pleurocapsa* cells below 48°C and also on masses of *G. terebriformis* which are washed downstream (Wickstrom and Castenholz 1985). Combined nitrogen may be limiting in the flowing water over this portion of the mat, since both the *Calothrix* and *Pleurocapsa* are capable of nitrogen fixation. By comparing the drainways of similar springs with and without ostracods it has become obvious that the *Pleurocapsa/Calothrix* community is not only quite grazer-resistant, but is actually grazer-dependent. It does not occur when the ostracods are absent. In such spring outflows the *Geitlerinema* (*Oscillatoria*) *terebriformis* cover extends downstream to about 35°C.

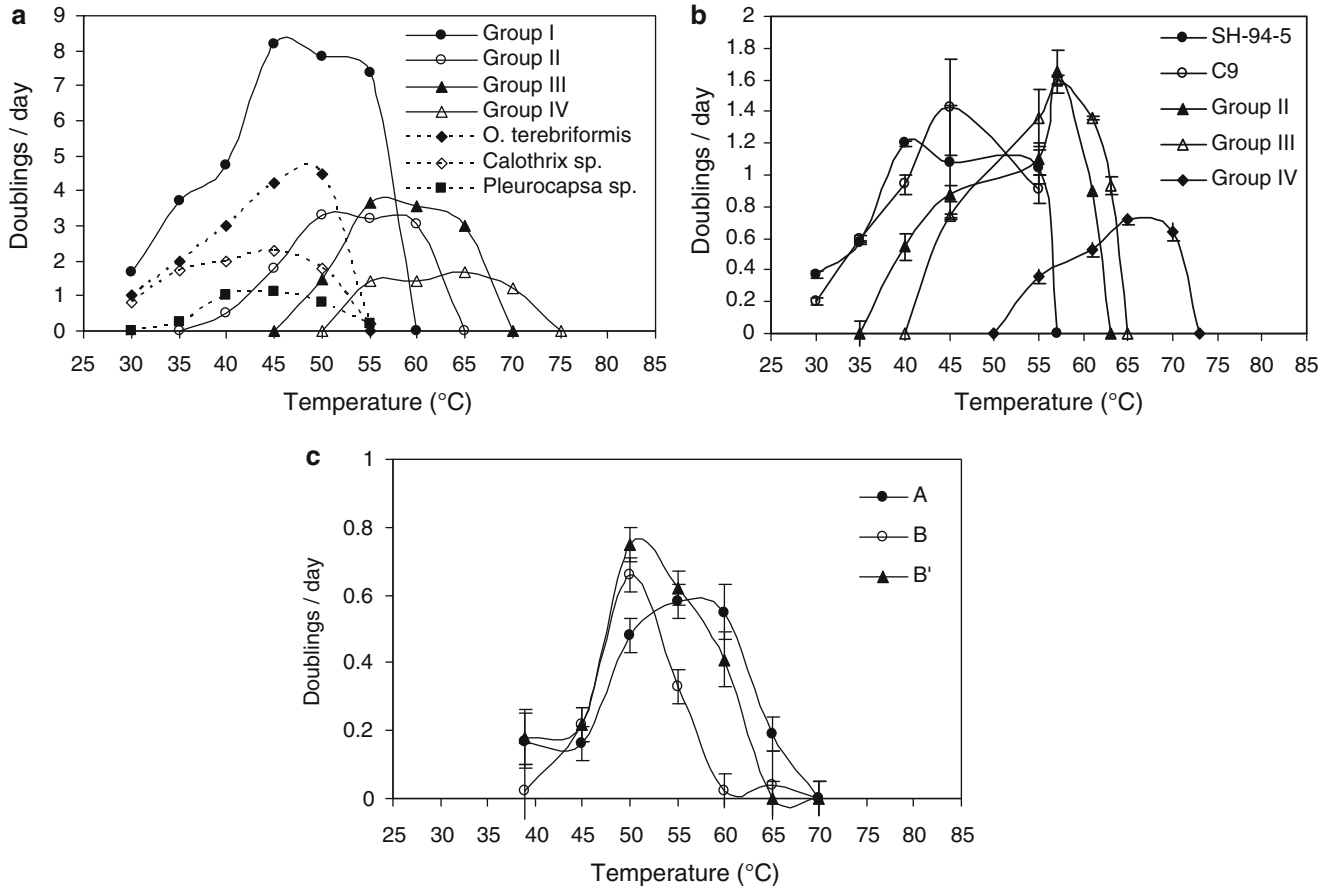


Fig. 3.4 Effect of temperature on growth rates of cyanobacterial isolates from (a, b) Hunter's Hot Springs, OR: (**a**) from Peary and Castenholz (1964); (**b**) from Miller and Castenholz (2000); (**c**) Octopus Spring, YNP: Allewalt et al. (2006). Except as noted in (**a**) all strains are *Synechococcus* thermotypes

3.2.3.2 Mushroom Spring and Octopus Spring, Yellowstone National Park

Mushroom Spring and Octopus Spring, located in the Lower Geyser Basin, YNP (Octopus Spring is in the White Creek drainage), have been studied extensively by T.D. Brock, Castenholz, Miller, Ward, their students and many others since the late 1960s. These are alkaline springs (pH ~8.3) of volcanic origin. Like Hunter's Hot Spring, the major ions are sodium (~13 mM) chloride (~8.2 mM), bicarbonate (~4.8 mM) and silicate (~1.8 mM). Unlike Hunter's Hot Spring, these springs have very low sulfate content (~0.3 mM) (Brock 1978; Papke et al. 2003). Until the mid-1990s, work was primarily conducted at Octopus Spring, but a major disturbance event (likely a hailstorm) that temporarily destroyed the Octopus Spring mat precipitated a shift to focusing primarily on nearby Mushroom Spring in the years since. Here we describe work done mainly at Mushroom Spring since that time (see Ward and Castenholz (2000) for a comparable description of the Octopus Spring landscape). These two springs are highly similar in their microbiota (compare Ward et al. 1998 and 2006). One interesting difference between these two habitats is the cooler source pool (see below); another is the cycling of temperature in Octopus Spring, which exposes sites in the effluent channel to shifts in temperature of ~10°C every 5–10 min (Miller et al. 1998). This may have interesting consequences for cyanobacterial diversity and its role in stabilizing oxygenic photosynthesis (see Figure 7 in Ward and Castenholz 2000).

A landscape view of Mushroom Spring and its cyanobacterial features is shown in Fig. 3.2a. As the source pool temperature (~70°C) is cooler than the upper temperature limit for cyanobacteria (~73–74°C), it is lined with a several millimetre thick translucent mat, which is more yellow-green at the surface and dark-green a few millimeters below the surface (Fig. 3.2b). This mat is comprised of *Synechococcus* cells resembling *S.* cf. *lividus* (Fig. 3.2c). As the source water flows into the effluent channels and cools downstream, these yellow-green and dark-green layers compress from a several millimeter thick translucent mat at ~68°C to a <1 mm-thick upper green layer at ~65°C and below (Figs. 3.2b and 3.5a). *Synechococcus* thermotypes have also been found in these systems (see Sects. 3.3.2 and 3.4.1.1). At temperatures

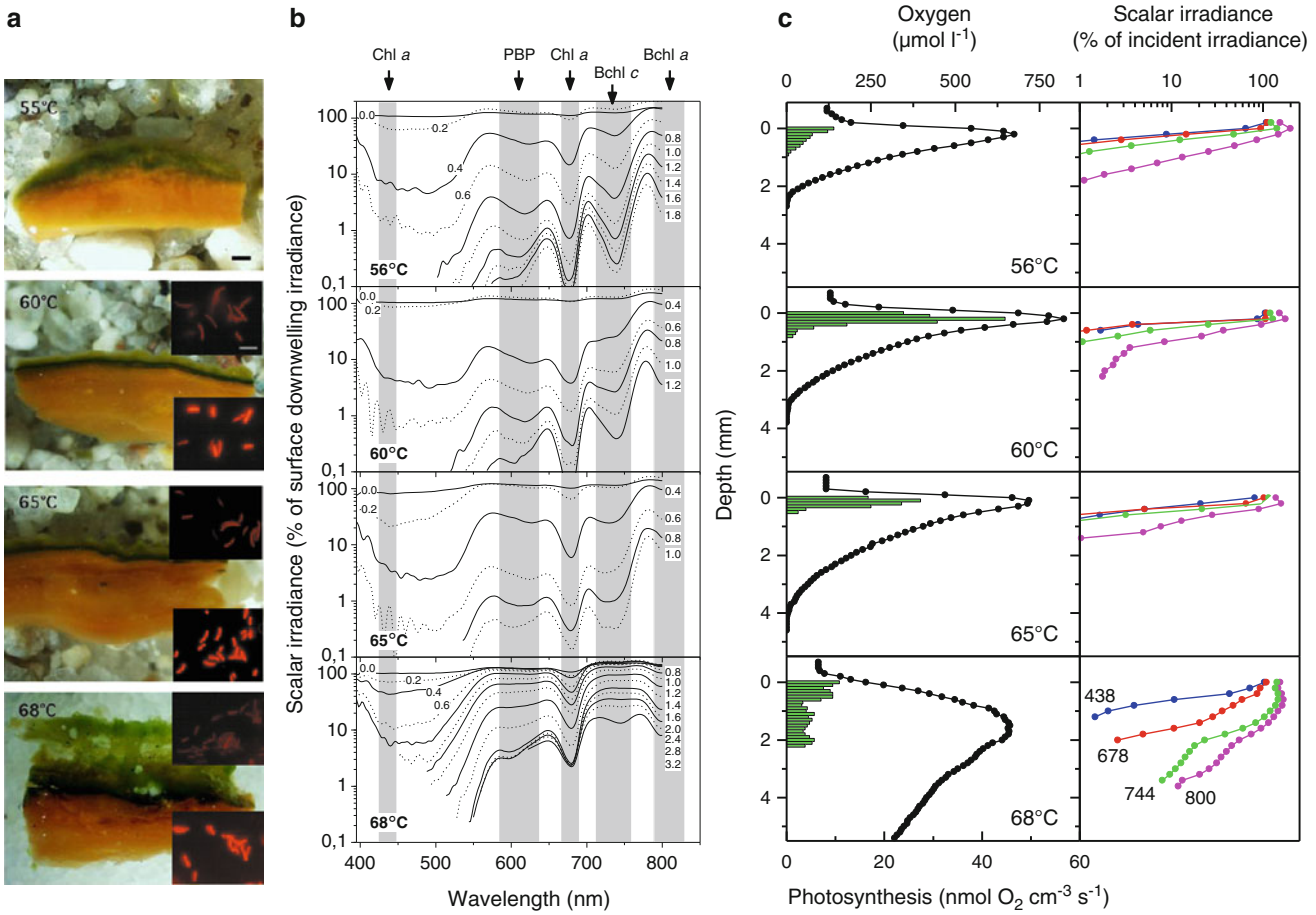


Fig. 3.5 Vertical aspect of *Synechococcus* mats in Mushroom Spring, YNP: (**a**) Cross sections of mat samples collected at 55°C, 60°C, 65°C and 68°C and autofluorescence photomicrographs of *Synechococcus* cells in the surface and subsurface parts of the top-green mat layer. *Scale bar* indicates 10 μm. (**b**) Vertical profiles of scalar irradiance of light of different wavelengths with depth (indicated in mm next to *lines*; *PBP* phycobiliprotein). (**c**) Vertical microsensor profiles of oxygen (*black line* and *points*), oxygenic photosynthesis (*green bars*) and penetration of light of specific wavelengths (From Ward et al. 2006)

between ~68°C and ~50°C, the *Synechococcus* cells in the upper yellow-green layer autofluorescence more dimly than those in the dark-green underlayer (Fig. 3.5a). Orange undermat layers are comprised largely of FAPs (e.g. *Chloroflexus* spp. and *Roseiflexus* spp.), as judged by fluorescence *in situ* hybridization with 16S rRNA probes (Nübel et al. 2002). These two types of FAPs are about equally abundant at 68°C, but *Roseiflexus* spp. predominate at lower temperatures. Overall mat thickness increases to up to several centimeters as temperature decreases along the effluent channel, though mat formation and decomposition are probably maximized in the upper few millimeters (Brock 1978; Ward et al. 1987).

Below approximately 58°C *Leptolyngbya* (*Phormidium*) spp. may be found together with *Synechococcus* spp., where they are especially obvious in reticulate features (Fig. 3.2d, e), streamers (in strong flow) and raised columns or pinnacles (in quiescent pools; Fig. 3.2f). *Leptolyngbya* (*Phormidium*)-dominated mats are usually orange in summer (Norris et al. 2002). The laminated mats and conical structures have been extensively studied as analogs of their fossil equivalents, planar stromatolites and the pinnacled Conophyton forms (see Walter et al. 1972; Brock 1978; Awramik and Vanyo 1986; Vanyo et al. 1986). Below approximately 43°C the larvae and adults of ephydrid flies graze upon the cyanobacterial mat (Fig. 3.2d inset). The thermophilic ostracod described in Sect. 3.2.3.1 occurs mainly in hot springs of the Great Basin region (e.g. Hunter's Hot Springs), and has not been found in any YNP springs. In the cooler waters further downstream (~40°C-ambient), N_2-fixing *Calothrix* forms brownish scytonemin-containing mats adhering to the siliceous substratum both in the flow and on the moist edges of the effluent (Fig. 3.2d, g).

3.2.3.3 White Creek

White Creek is a tributary of the Firehole River which drains many thermal features of the Lower Geyser Basin of Yellowstone National Park, including Octopus Spring and

Great Fountain Geyser (Fig. 3.3a). Its chemistry is similar to the primary hot springs it drains with respect to pH (~8.2), silicate (~4.2 mM) and sulfate (~–0.1 to 0.2 mM), but it has lower concentrations of sodium (~4.3 mM) and chloride (~1.4 mM), presumably due to dilution by surface stream water (Miller, unpublished data). White Creek, as a high volume and velocity stream, is characterized by a much shallower thermal gradient (2°C per 100 m) than the primary thermal features (Miller et al. 2009a). Consequently, cyanobacterial mat communities are distributed along an approximately 1.5 km stretch of the channel with mean annual temperature ranging between approximately 72°C and 39°C (including a large hot spring that serves as one of the primary upstream sources of geothermally heated water).

At temperatures greater than about 55°C, laminated mats of *Synechococcus* and FAPs similar to those shown in Fig. 3.2 predominate. Between ~55°C and 64°C, large cells of the heterocyst-forming *Chlorogloeopsis* are also occasionally evident. At lower temperatures, between ~39°C and 55°C, streamer mats composed of *Fischerella (Mastigocladus) laminosus*, other cyanobacteria and FAPs are observed (Fig. 3.3b, c). Few sites in YNP harbour large amounts of *F. laminosus* biomass, though this cyanobacterium has been obtained from many locations in enrichment cultures, and the generally low concentrations of combined N that are measured in this region of White Creek (Miller et al. 2006, 2009b, unpublished data) likely contribute to its success. Intercalary heterocysts are evident in *F. laminosus* trichomes, and mats actively fix N_2 as assayed by acetylene reduction (Stewart 1970; Miller et al. 2006). Members of the *F. laminosus* population have diverged in thermal performance (i.e. different thermotypes), and distribution of this ecological variation is tightly associated with environmental temperature (Sect. 3.4.2; Miller et al. 2009a). Particularly near the upstream population boundary, trichomes of *F. laminsosus* are tightly associated with adhered *Synechococcus*, whereas at downsteam locations other cyanobacteria are more evident.

3.3 Distribution of 16S rRNA–Defined Taxa

The contemporary distributions of cyanobacteria in geothermal habitats are potentially shaped by various factors, including dispersal limitations that result in biogeographic patterns and environmental heterogeneity (i.e., habitat sorting). We begin with a global perspective before appraising the contribution of parameters related to effluent flow away from source pools (e.g., temperature) to the realized niches (i.e. actual *in situ* distributions, as opposed to the fundamental or potential niches defined by laboratory studies) of thermophilic cyanobacteria in local settings.

3.3.1 Biogeographic Distribution

A current focus of microbial ecology has been on the issue of how microorganisms are distributed at a variety of geographic scales (reviewed by Martiny et al. 2006). Determining the spatial structure of microbial diversity is a prerequisite for developing an understanding of the processes that shape distribution patterns. A question of particular interest is whether dispersal barriers to migratory gene flow exist for microorganisms, i.e., whether microbial populations are geographically isolated (Finlay 2002; Papke et al. 2003; Whitaker et al. 2003). Genetic divergence of populations at neutral loci is expected to increase with physical distance in the absence of significant migration (Wright 1943), because local selective sweeps (Sect. 3.4.1.2) and genetic drift remove variation within, but not between, populations. Although it has long been recognized from morphological surveys of microbial mat communities from geothermal habitats that certain cyanobacteria exhibit restricted geographic ranges (Sect. 3.2.1; Castenholz 1996; Ward and Castenholz 2000), advances in molecular biological approaches for investigating microbial diversity *in situ* have greatly enhanced our appreciation of this biogeographic structure (Fig. 3.6). Papke et al. (2003) provided the first study of hot spring cyanobacterial 16S rRNA diversity at global and regional scales. For environmental samples from North America, Japan, New Zealand and Italy, the authors (i) confirmed that taxa vary in their breadth of distribution, (ii) reported that patterns of taxon distribution could not be explained by spring geochemistry, and (iii) concluded that allopatric divergence of geographically isolated populations generally plays a key role in the origins and maintenance of diversity. Surveys of molecular diversity from other geothermal regions (as well as numerous unpublished environmental sequence data in public databases) have provided a rich comparative data set that generally corroborates the conclusions of Papke et al. (2003). Included in these surveys were collections from Australia (Anitori et al. 2002), Greenland (Roeselers et al. 2007), Jordan (Ionescu et al. 2010), The Philippines (Lacap et al. 2007), Thailand (Jing et al. 2005; Portillo et al. 2009; Sompong et al. 2008; Lau et al. 2009), and Tibet (Lau et al. 2006, 2008, 2009). Below, we summarize the distribution patterns of representative taxa.

Many thermophilic cyanobacteria exhibit severely restricted geographic distributions (Table 3.1, Fig. 3.6). Perhaps the most notable example is the *Synechococcus* A/B clade (defined in Sect. 3.4.1). Definitive evidence for its presence, based on the recovery of environmental sequences and/or sequenced laboratory isolates, currently exists only from North American hot springs (e.g. Ward et al. 1998; Miller and Castenholz 2000; Papke et al. 2003; Allewalt et al. 2006). There is morphological evidence for the presence of high-temperature forms of *Synechococcus* in hot springs of China

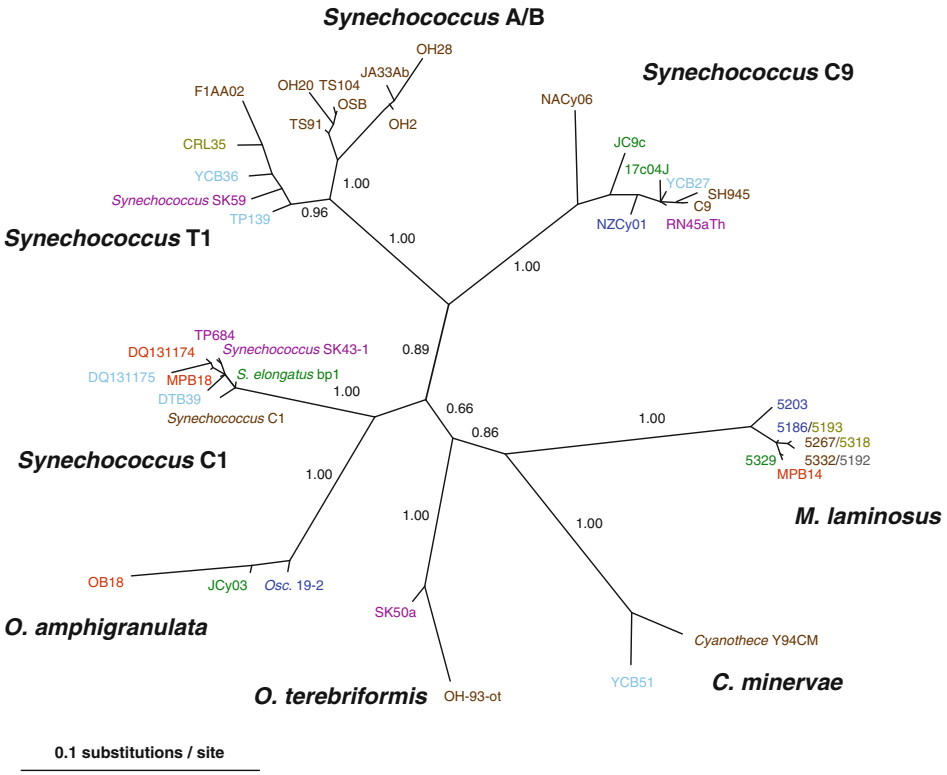


Fig. 3.6 Bayesian phylogeny (Huelsenbeck et al. 2001) of the 16S rRNA gene emphasizing the geographic distributions of representative thermophilic cyanobacteria. Sequences were obtained from: North America (*brown*); Japan (*green*); the Philippines (*orange*); Thailand (*purple*); Tibet (*light blue*); New Zealand (*dark blue*); and Central/South America (*olive*). Two independent Metropolis-coupled MCMC chains of 1,000,000 generations each were run in Mr. Bayes according to a GTR+G+I model of sequence evolution. Chain convergence was assessed by the average standard deviation of split frequencies. The consensus diagram shown was produced from a sample of 1,800 trees following discard of 10% of sampled trees as burn-in. Clade credibility values at nodes represent the posterior probability that the monophyly of a clade is supported by the data. For clarity, these values have been removed within individual clades

(Yun 1986), Thailand (Sompong et al. 2008), Central and South America and possibly Africa (Castenholz 1996), but the genotypes of these organisms are not yet known. Interestingly, molecular evidence has been obtained from several studies for a sister clade of the A/B clade (described as "Lineage T1" in Lau et al. 2009) that exhibits a far wider geographic distribution (Fig. 3.6). Environmental sequences of this group have been recovered from microbial mat communities from Tibet (Lau et al. 2006, 2008, 2009; Yim et al. 2006), Thailand (Jing et al. 2005; Sompong et al. 2008), Jordan (Ionescu et al. 2010), Costa Rica (unpublished GenBank sequence accession no. EF545646) and YNP. The YNP T1-like sequences are from lithified coniform structures in Black Sand Pool (see inset to Fig. 3.2f) (Lau et al. 2005), mesothermic microbial mat from the margins of an unidentified hot spring pool (unpublished GenBank sequence accession no. FJ885003) and from 38°C mat at Rabbit Creek, where it appears to be rare (an estimated ~0.5% of the community; Miller et al. 2009a). In addition, representatives of the T1 group have been isolated in laboratory culture from a 36°C enrichment of material collected from the Kotel'nikovskii hot spring of the Baikal rift (*Synechococcus* sp. 0431; Sorokovikova et al. 2008) as well as from San Kamphaeng hot spring in Thailand (Sompong et al. 2008). Although environmental data were not reported for many of the sites from which these sequences and strains were derived, the trend appears that members of the T1 group are found primarily at lower temperatures than are members of the A/B group. If this is the case, then an intriguing possibility is that tolerance of lower temperatures than in the A/B group might help to explain the broader distribution and apparently greater dispersal capabilities of the T1 group. It is also of interest whether members of the T1 group occupy higher temperature niches in regions where representatives of the A/B clade are absent.

Another example of a thermophilic cyanobacterium with an apparently very restricted distribution is *Cyanothece* (*Synechococcus*) cf. *minervae* (Table 3.1). This phycoerythrin-producing cyanobacterium is obvious microscopically in YNP collections, most notably from below about 62°C at Mammoth Hot Springs (e.g. Minerva Terrace, from which its name is derived) and from Chocolate Pots Spring, and it has

been isolated in laboratory culture from White Creek, from a hot spring near Lone Star Geyser and from South Harney Hot Springs in Oregon (Miller, unpublished data). With respect to evidence from environmental sequences, the only other location from which it has been recovered is from near Yibbug Caka, Tibet (64°C; Lau et al. 2009).

A taxon with an unusual distribution is *Geitlerinema* (*Oscillatoria*) cf. *terebriformis* (Table 3.1; Fig. 3.6). Molecular evidence for thermophilic members of this taxon comes from North America (Hunter's Hot Springs, OR, northern California, and Nevada, Idaho, western Montana, Alum Rock Park, CA), Saudi Arabia and Thailand (Sompong et al. 2008). Isolates from the western USA and Saudi Arabia have essentially identical 16S rDNA sequences (T.B. Noriis, unpublished data). However, close relatives of *G.* cf. *terebriformis* (>97% 16S rRNA sequence identity) have been found in a variety of freshwater locations (e.g. Lake Alexandrina, Australia, and a limestone sinkhole in Mexico). This suggests the possibility of a relatively recent evolutionary origin of thermophily in the group. Thermophilic, sulphide-tolerant *Leptolyngbya* (*Oscillatoria*) cf. *amphigranulata* is apparently restricted to New Zealand (Garcia-Pichel and Castenholz 1990), Japan (Papke et al. 2003) and the Philippines (Lacap et al. 2007) (Fig. 3.6).

By contrast, many taxa appear to be more widely distributed: These include the *Synechococcus* C1 and C9 groups, and the heterocyst-forming *Fischerella* (*Mastigocladus*) *laminosus* (Table 3.1, Fig. 3.6). Typically, corroborating evidence for their greater breadth of distribution also exists from the microscopic observation of environmental samples and from culture collections (see above).

There are good reasons, however, to believe that dispersal barriers to migration exist even for the most widely distributed thermophilic cyanobacteria. Miller et al. (2007) observed a strong positive correlation between the amount of genetic differentiation between populations of the cosmopolitan bacterium *F. laminosus* and the physical distance which separates them. This result meets the prediction of the isolation-by-distance model (Wright 1943), in which the probability of dispersal decreases with increasing distance. Miller et al. (2006) explicitly estimated the migration rates between relatively close White Creek and Boiling River, YNP populations of *F. laminosus*, which are separated by approximately 50 km. These populations exhibit considerable genetic differentiation based on sequence data from four loci involved in nitrogen metabolism (*nifH*, *narB*, *nirA*, *devH*), with no shared genotypes between them. However, the degree of genetic differentiation (i.e., $_{FST}$) of these populations could in principle be explained by an equilibrium between migration and genetic drift, rather than simply by geographic isolation (i.e., the absence of migration). Using the isolation-with-migration model (Nielsen and Wakeley 2001; Hey and Nielsen 2004) to distinguish between these possibilities, migration between the populations was undetectable, supporting the conclusion that the White Creek and Boiling River populations were recently geographically isolated (Miller et al. 2006).

Although these results suggest that successful migration across even moderate distances should be considered an extremely low probability event, even for "strong" dispersers, these events still clearly happen on a contemporary time scale. For example, identical multi-gene haplotypes of *F. laminosus* have been recovered from geographically distant locations including Montana (USA), Chile and Oman (Miller et al. 2007; Ionescu et al. 2010). A possible explanation for the geographically wide distribution of these *Fischerella* genotypes, is the fact that this cyanobacterium tolerates desiccation and freezing, produces akinete-like cells and also grows slowly even at non-thermal temperatures (25–30°C), which may allow it to exist in many "stepping stone" aquatic habitats.

3.3.2 Distribution Along Effluent Flow Path

Although chemistry may also change as water flows away from the source pool, temperature is a primary organizer of cyanobacterial diversity along hot spring outflow channels. The temperature dependence of the distributions of *Synechococcus* A-like and B-like cells in Octopus Spring (Ferris and Ward 1997; Ward et al. 1998; Ward and Castenholz 2000) and Mushroom Spring (Ward et al. 2006 based on denaturing gradient gel electrophoresis (DGGE) banding patterns remains a prime example of ecotypic differentiation in the microbial world, with members of the A-like and B-like groups generally more abundant at higher and lower temperatures, respectively. Specifically, 16S rRNA genotype variants A″, A′, A, B′ and B are distributed progressively from ~72–74°C to ~50°C along the effluent flow path (see Figure 4 of Ward and Castenholz 2000). Similarly, A-like and B-like 16S rRNA genotypes were retrieved by PCR amplification and cloning from ~60°C to ~50°C regions of Hunter's Hot Springs, OR (Papke et al. 2003).

More recently, DGGE has also been used to investigate the cyanobacterial diversity of the microbial mats that develop at lower mean temperature than that mentioned above. Norris et al. (2002) observed largely similar communities for 40–47°C mats from Octopus Spring and a tributary of Rabbit Creek, respectively, including members of the *Synechococcus* C9 and OS Type P groups (see Ferris et al. 1996). Lau et al. (2005) specifically examined topographical patterns of diversity of and around the raised structures at Black Sand Pool, Yellowstone NP. These structures, believed to be produced primarily by phototactic filamentous cyanobacteria, are often conspicuous features of alkaline hot spring microbial mat communities at lower temperature (Fig. 3.2f). As found by Norris et al. (2002), the raised structures contained *Synechococcus* OS Type P and C9 cells.

An additional DGGE band sequence was related to a filamentous cyanobacterium that has been isolated in laboratory culture from low temperature mats from Octopus Spring (Norris et al. 2002; cyanobacterium OLI-01) and a hot spring in Fairy Creek meadows, NP (Bosak et al. 2009; *Leptolyngbya* sp. FYG). Two other DGGE band sequences appear to belong to the *Synechococcus* "T1" sister group of the *Synechococcus* A/B clade (see above). In contrast, members of the *Synechococcus* B group were recovered from the surrounding nonlithified mat.

Since the publication of Ward and Castenholz (2000), advances in high-throughput DNA sequencing technologies (e.g., Margulies et al. 2005) have enabled more extensive sampling of spatial distribution of microbial diversity along thermal gradients. Miller et al. (2009a) employed a barcoded pyrosequencing approach using PCR amplification to target the V3 region of the bacterial 16S rRNA gene to investigate community structure along the thermal gradients of White Creek and Rabbit Creek. It should be noted that a limitation of using the V3 sequence is reduced phylogenetic resolution for some taxa, particularly among members of the diverse *Synechococcus* B group, which have identical sequences in this region. Approximately one-third of the nearly 34,000 total sequences collected from ten sites along each gradient were of cyanobacterial origin. Of these, a combined total of 41 unique V3 sequence signatures (termed operational taxonomic units (OTUs) in Miller et al. 2009a) were recovered from the two streams. By contrast with what was found for Mushroom Spring above, the FAPs were almost exclusively *Chloroflexus* spp. along the entire White Creek thermal gradient (Miller et al. 2009b).

As expected, A-like *Synechococcus* lineages dominated at the highest temperature sites, with the same two OTUs together comprising a majority of the entire microbial communities at both creeks (Fig. 3.7). In White Creek, B-like *Synechococcus* lineages dominated between 58°C and 64°C. In Rabbit Creek, the 63–64°C region of the thermal gradient was primarily occupied by a member of the A-like *Synechococcus* group (OTU 53) that was very rare at White Creek. The breadth of the realized distribution of the B-like *Synechococcus* OTU also differed between channels. At Rabbit Creek, these were the most abundant cyanobacteria between approximately 47°C and 61°C, whereas, at White Creek, *Fischerella laminosus* and OS Type P-like *Synechococcus* predominated at temperatures below about 55°C (Fig. 3.7) (Miller et al. 2009a). The springs differ greatly in the availability of combined nitrogen at temperatures below ~55°C (Miller et al. 2009b), and this may help to explain the presence of the heterocyst-forming, nitrogen-fixing *F. laminosus* at White Creek (instead of B-like *Synechococcus* populations) and its apparent absence at Rabbit Creek.

The clearly resolved range boundaries among different *Synechococcus* lineages provide additional insights into the ecological interactions among these bacteria. The realized niches observed for *Synechococcus* OTUs are more narrow than the temperature ranges permissive for growth of related strains in the laboratory (Miller and Castenholz 2000; Allewalt et al. 2006), and this disparity between the realized and potential niches of representative strains can be attributed to competition in regions of overlap.

At Rabbit Creek, the lowest temperature site was dominated by an unidentified filamentous cyanobacterium that was also detected in Octopus Spring lower temperature mat (Norris et al. 2002; DGGE bands OL6 and OL7). While other taxa were comparatively rare, significant abundances (<1–5% of the total microbial community) for OS Type I, OS Type P and *Synechococcus* C9 groups were observed at some sites (Fig. 3.7).

3.4 Ecology of Well-Studied Hot Spring Cyanobacterial Communities

3.4.1 Mushroom Spring and Octopus Spring *Synechococcus* Mats

The 50°C to 73–74°C *Synechococcus* mats of alkaline siliceous hot springs have been studied for many decades and serve as model systems for understanding the composition, structure and function of microbial communities (Brock 1978; Ward et al. 1998, 2006, 2008, 2011, 2012; Ward and Castenholz 2000). Sections 3.2.3.2 and 3.3.2 showed the positioning of these communities relative to other cyanobacteria found at lower temperatures in these and other springs and introduced the *Synechococcus* A/B-lineage 16S rRNA genotypes that predominate. Here, we update our understanding of linkages among genetic, taxonomic and functional diversity based on studies since Ward and Castenholz (2000).

3.4.1.1 Evidence of Adaptive Differences Among Isolates Relevant to These Mat Systems

The distribution of closely related 16S rRNA variants along the effluent flow path (genotypes A″, A′, A, B′ and B from ~72–74°C to ~50°C (Fig. 3.8a); Ferris and Ward 1997; Ward et al. 2006; Miller et al. 2009a) and vertical dimension (at ~60°C, genotype B′ above genotype A; Ramsing et al. 2000; see Figure 6 in Ward and Castenholz (2000)) led to the hypothesis that *Synechococcus* spp. with these genotypes likely arose through adaptation to parameters that vary along the associated gradients of temperature, light and chemistry (Ward 1998). Allewalt et al. (2006) succeeded in cultivating *Synechococcus* strains with A, B′ and B 16S rRNA genotypes (Table 3.1) and used them to test the hypothesis of adaptation by demonstrating that their temperature preferences were consistent with the *in situ* distributions of

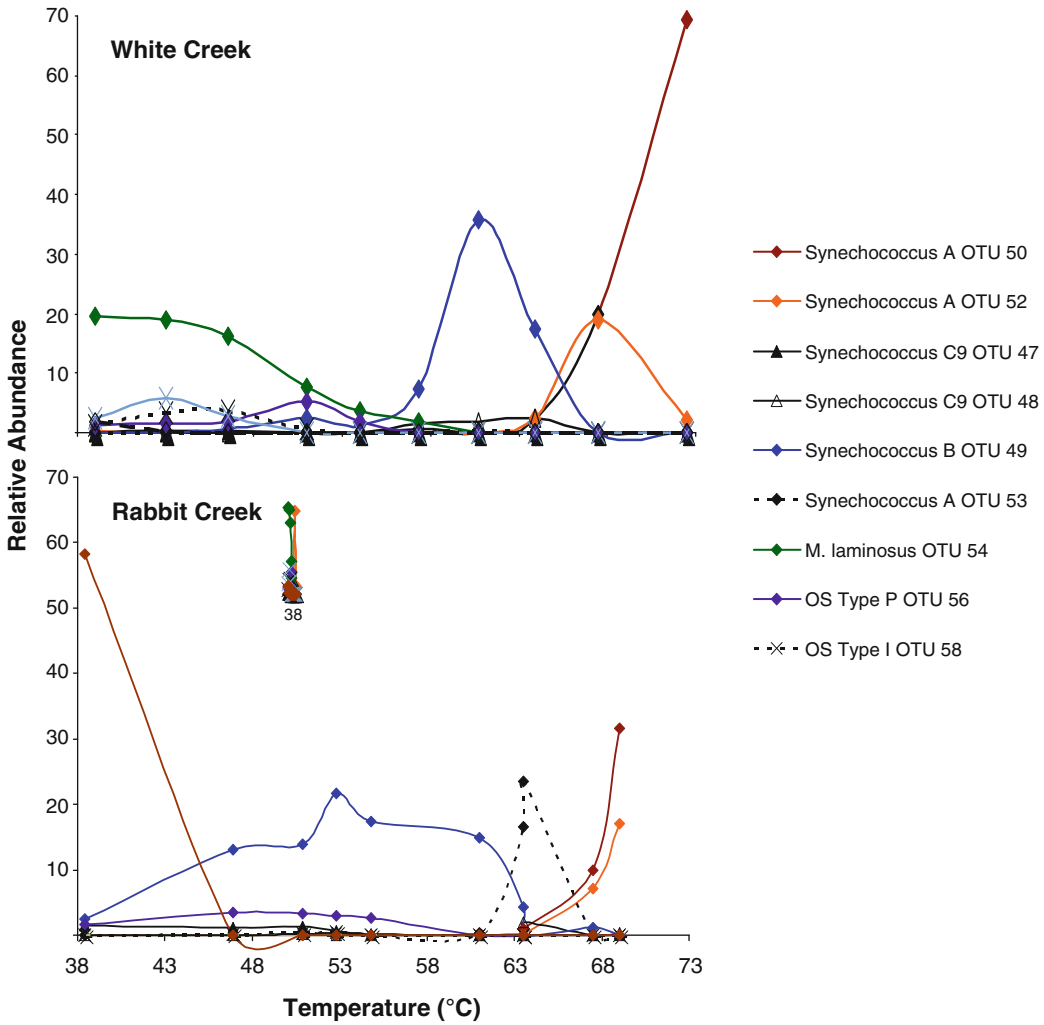


Fig. 3.7 Distribution of major cyanobacterial taxa along the thermal gradients of White Creek and Rabbit Creek. Relative abundance refers to the percentage of sequences of an OTU recovered from a particular environmental sample (Data from Miller et al. 2009b)

their 16S rRNA genotypes (Fig. 3.4c). As mentioned above, Miller and Castenholz (2000) had already demonstrated this for Oregon A/B-like *Synechococcus* strains. Molecular evidence of adaptation has been observed among the Oregon strains for the Calvin cycle enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). The RuBisCO enzyme of *Synechococcus* strain OH28, which is capable of growth at 70°C (Miller and Castenholz 2000), exhibits enhanced thermostability (~7°C greater estimated denaturation temperature in circular dichroism thermal scans) relative to either the native RuBisCOs of less thermotolerant strains or inferred ancestral *Synechococcus* RuBisCOs constructed by sequential site-directed mutagenesis (Miller, unpublished data).

Complete genome sequences have been obtained for *Synechococcus* spp. strains A and B′ (Bhaya et al. 2007) and comparative analyses revealed differences in nutrient acquisition and storage. Specifically, the B′ strain has genes that enable it to use phosphonate as a source of phosphorus (Adams et al. 2008), as well as genes for producing and degrading cyanophycin, which may be involved in storage of nitrogen. These observations may foretell of decreasing availability of phosphate and fixed nitrogen forms downstream in the effluent channel. Genomic analyses also revealed that both strains have the potential to fix N_2, which is interesting in light of the prior work of Stewart (1970) and Wickstrom (1980), which led these authors to the suggestion that *Synechococcus* in such mats do not have the capability to fix N_2. *In situ* expression of *nif* genes and proteins and nitrogen fixation was demonstrated (Steunou et al. 2006, 2008) and will be described in more detail below.

Synechococcus populations at the mat surface (Fig. 3.5a) have distinctly different autofluorescence than those residing ~400–700 μm beneath the mat surface suggesting acclimation

and/or adaptation to lower light intensity and/or differences in light quality (Fig. 3.5b, c). The observations by Ramsing et al. (2000) of (i) the co-occurrence of a genetically distinct *Synechococcus* population (16S rRNA genotype A) with the deeper, more fluorescent population at a ~60° mat site, and (ii) unique cell reorientation of the deeper population at the brightest part of the light cycle, provided further evidence consistent with the hypothesis of differential light adaptation. The light relations of B-, B′- and A-like *Synechococcus* isolates have been studied. While all isolates could photosynthesize at up to 1,100 µmol photon m^{-2} s^{-1} and grow at up to at least 385 µmol photon m^{-2} s^{-1} (Allewalt et al. 2006), the B′ isolate was reportedly unable to maintain growth for more than 5 days at 400 µmol photon m^{-2} s^{-1} (Kilian et al. 2007). Even at 200 µmol photon m^{-2} s^{-1} this isolate reduced levels of phycobilisomes and chlorophyll and increased levels of carotenoids. The interpretation of these results is complex. The *Synechococcus* sp. strain B′ isolate might live in the upper photic zone and tolerate high light intensity. *In situ* it never experiences continuous high light for many days. Furthermore, surface *Synechococcus* populations appear to shut down oxygenic photosynthesis in the brightest part of the day (Ramsing et al. 2000). Alternatively, this strain might reside in lower parts of the photic zone and be low-light adapted. B′-like *Synechococcus* populations are also found deeper in the 60°C mat (Ramsing et al. 2000; Becraft et al. 2011) and there is evidence that photosynthesis maximizes in deeper layers at the highest light intensities (Ramsing et al. 2000). Clearly, more information about growth as a function of light intensity and/or quality is needed to resolve whether light adaptations occur in thermophilic *Synechococcus* spp.

3.4.1.2 Nature of *Synechococcus* Species in These Mats

At 65°C and 68°C sites, single 16S rRNA genotypes A and A′, respectively, occur at all depths in the photic zone (Ward et al. 2006). The question arose as to whether the phenotypically distinct *Synechococcus* populations at different mat depths at these temperatures (Fig. 3.5a) represented a single genetic population (i.e., species) acclimated to differing environmental conditions or >1 species adapted to different vertical microenvironments and so closely related that they could not be resolved using the slowly evolving 16S rRNA molecule. Ferris et al. (2003) showed that a more rapidly evolving genetic locus, the internal transcribed spacer (ITS) sequence separating 16S and 23S rRNA genes, could resolve the A′-like *Synechococcus* populations occurring at 68°C into two, more closely related genetically distinct populations, which occur at surface or sub-surface depths. However, at ~65°C the differently fluorescing A-like *Synechococcus* populations could not be resolved by either 16S rRNA or ITS sequence variation. These observations raised the question of how much molecular resolution is needed in order to discern all *Synechococcus* species in the mats. We have examined many protein-encoding genes in both single-locus and multi-locus analyses to address this question, as will be described below. However, to understand the results of these analyses, it is necessary to first consider the central question of how individual genetic variants are grouped into species populations. The realization that the origin of differently adapted *Synechococcus* species in these mats is best described by the Stable Ecotype Model of species and speciation (Ward and Cohan 2005) precipitated the development of approaches that have provided a way to answer to this question.

In the Stable Ecotype Model (Cohan and Perry 2007), ecotypes, known also as ecological species (*sensu* Van Valen 1976), are conceptualized as populations of ecologically interchangeable variants that differ genetically because of ecologically neutral mutation and/or recombination events that accumulate uniquely in organisms occupying distinct niches. Ecotypes evolve as natural selection periodically favours the most-fit variant among those capable of inhabiting a niche. Periodic selection thus reduces genetic diversity within an ecotype, which rises anew as ecologically neutral mutations accumulate in the survivor of the most recent periodic selection event. When variants arise that are ecologically different than other variants in the ecotype, they initiate new populations because they are not affected by the periodic selection events affecting the parent population; they diverge and eventually evolve into a new and distinct ecological species (i.e. ecotypes). Such populations can be demarcated from molecular sequence variation, since each species accumulates different genetic variants. Koeppel et al. (2008) developed a Monte Carlo evolutionary simulation (Ecotype Simulation) based on the Stable Ecotype Model that determines the order and frequency of periodic selection and ecotype formation events and the number of ecotypes that best explains the evolution of a lineage as reflected by gene phylogenies. Consequently, this algorithm predicts how individual molecular variants are grouped into ecological species populations. A test of Ecotype Simulation using the ITS results mentioned above demonstrated that the putative *Synechococcus* ecotypes predicted by Ecotype Simulation do have distinctive ecology (i.e. unique distributions along flow and vertical gradients; (Fig. 3.8b) see Ward et al. 2006). Ecotype simulation analysis of protein-encoding loci has revealed the existence of on the order of 13–14 ecological species of *Synechococcus* within both the A and B′ 16S rRNA genotypes, and fine-scale distribution patterns, gene expression patterns and population dynamics in response to environmental alteration confirm the ecological distinctions of these predicted ecotype populations (Melendrez et al. 2011; Becraft et al. 2011; Ward et al. 2011) (Fig. 3.8c). The number of predicted ecotypes rises with extent of sampling of a population and with the molecular resolution of

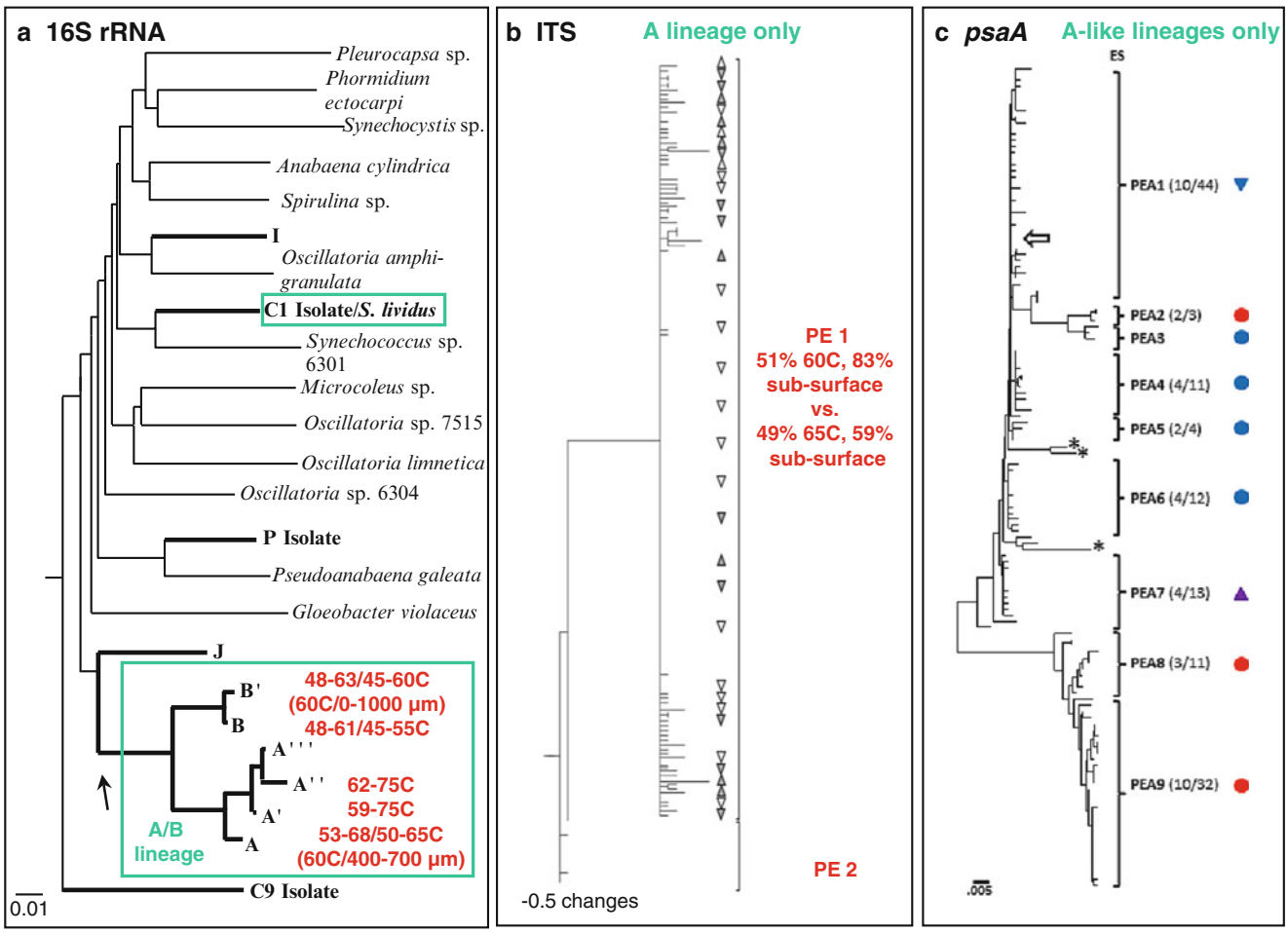


Fig. 3.8 Phylogenetic trees showing *Synechococcus* putative ecotypes (*PE*) detected by Ecotype Simulation analysis of sequence data: (**a**) Partial 16S rRNA gene; (**b**) 16S–23S rRNA ITS sequence; (**c**) Partial *psaA* gene. *Red text* indicates information about temperature and depth distribution of PEs. *Green boxes* in panel **a** highlight the *Synechococcus* A/B lineage and the sequence of the readily cultivated *S. lividus*. In (c) colors indicate temperatures (blue, 60°C; purple, 63°C; red, 65°C) and triangles point toward surface and sub-surface populations. (Data from Ward et al. 1998, 2006; Becraft et al. 2011)

the gene studied (Melendrez et al. 2011). Ecotypes are also detected in cultivation-independent multilocus sequence analysis, despite evidence that recombination exceeded mutation during the evolution of the *Synechococcus* A/B lineage (Melendrez et al. 2012). We estimate that between ~60°C and 65°C sites there are on the order of 30 *Synechococcus* ecotypes (i.e., ecological species), which are concealed by common morphology and 16S rRNA genotypes.

3.4.1.3 Metagenomic Analysis of Community Composition

Metagenomic sequences were obtained from the upper cyanobacterial layers of ~60°C, ~65°C and ~68°C regions of the Mushroom Spring mat and ~60°C and ~65°C regions of the Octopus Spring mat (Bhaya et al. 2007; Klatt et al. 2011). Based on phylogenetic similarity and oligonucleotide frequency analyses, these sequences assembled into eight well-defined clusters (Fig. 3.9a), one of which corresponds to A-like and B′-like *Synechococcus* populations. This population comprises about 30% of the genes in the metagenomes and, as in 16S rRNA analyses, A- and B′-like populations predominate at 65°C and 60°C, respectively (Klatt et al. 2011). Comparative analysis of these metagenomes and the *Synechococcus* spp. strain A and B′ genomes revealed that the isolates were closely related to native populations (Fig. 3.9b). Metagenomic clones with only one end sequence that was closely associated with the *Synechococcus* spp. strain A or B′ reference genome revealed (through the sequences at the other end of the same clone) differences

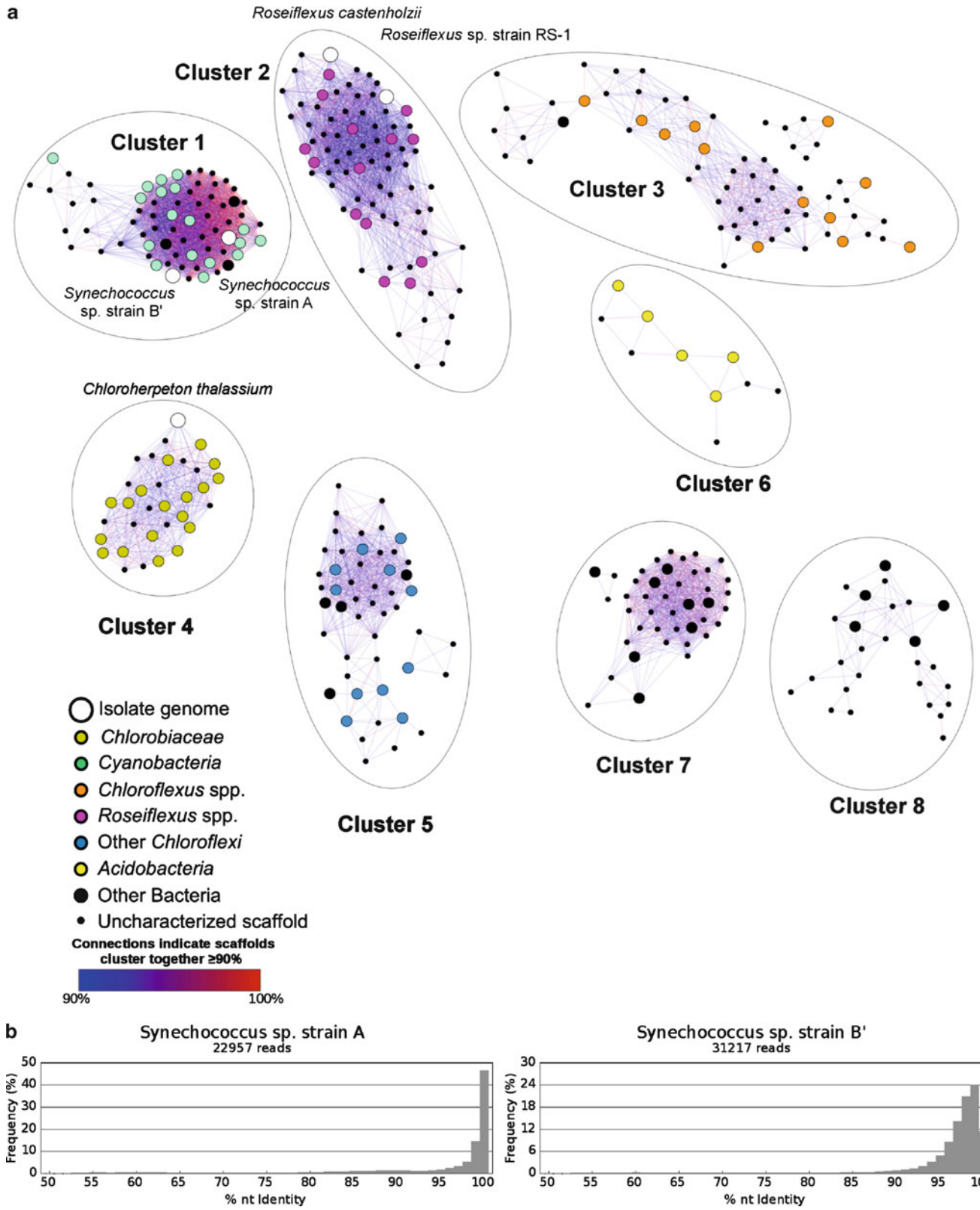


Fig. 3.9 Metagenomic analysis of 60°C and 65°C regions of Mushroom Spring and Octopus Spring mat top green layers: (**a**) Network map of core scaffold clusters observed in Celera assemblies. Scaffolds with similar oligonucleotide frequency profiles that group together in the same cluster in ≥90% of 100 trials are indicated by *connecting lines*, whose color reports the percentage that scaffolds group together as defined by the *scale bar*. Isolate genomes included in this analysis are indicated by *large white circles*, whereas metagenomic scaffolds that contain characterized phylogenetic marker genes are marked as *medium-sized circles* colored according to taxonomic grouping. *Large ovals* were drawn by hand to demarcate the different clusters. (**b**) Histograms of metagenomic sequences that can be associated confidently with the *Synechococcus* sp. strain A (*left*) or strain B′ (*right*) reference genome presented as a function of their % NT ID relative to the reference genome that recruited them in BLASTN analysis (From Klatt et al. 2011)

between cultivated and native *Synechococcus* populations. For instance, both A-like and B′-like populations appear to include organisms, which, unlike the cultivated strains, possess *feo* genes that might confer the ability to incorporate ferrous iron (Bhaya et al. 2007; Klatt et al. 2011). Fe^{2+} acquisition could be a heretofore unsuspected niche-determining characteristic. Genes closely related to those of other thermophilic cyanobacteria (e.g. *Thermosynechococcus elongates*, a member of the C1 *Synechococcus* lineage: see Papke et al. 2003) were not detected. This was consistent with their low abundance in Yellowstone cyanobacterial mats (Papke et al. 2003; Miller et al. 2009a; see Fig. 3.7).

Five of the other metagenomic clusters correspond to anoxygenic phototrophic bacteria, including *Roseiflexus*, *Chloroflexus*, a member of the Chlorobiales, *Candidatus* Chloracidobacterium thermophilum and a novel phototroph that is phylogenetically deep within Kingdom Chloroflexi (Klatt et al. 2011). Two other clusters appear to represent heretofore unknown community members that, based on current analyses, do not appear to contain genes associated with phototrophic metabolisms. Clearly, the complexity of phototrophs and other dominant members of these mat communities is greater than previously thought, especially when it is clear that these clusters may, like the *Synechococcus* cluster, contain numerous closely related species.

Metagenomes have also been obtained for the Mushroom Spring 60°C undermat and other Yellowstone geothermal features dominated by cyanobacteria, including, White Creek and Chocolate Pots Spring (Klatt et al. 2012b). These databases are beginning to broaden our understanding of connections between potential function and cyanobacteria in these geochemically different habitats.

3.4.1.4 *In Situ Synechococcus* Physiology

Oxygen microsensors have been used extensively to document oxygenic photosynthesis and the resulting oxygen profiles relative to the *in situ* realities of light in these mats (e.g. Ward et al. 2006) (Fig. 3.5b, c). Mats at temperatures ≤65°C have such compressed photic zones (Fig. 3.5a) that vertical profiles of oxygenic photosynthesis usually show only one depth at which photosynthesis maximizes (Fig. 3.5c). However, the more translucent mats at ~68°C show evidence of possible multiple photosynthetic maxima at different depths (Fig. 3.5c). Distinct photosynthesis maxima at different depths was also observed during a period of recovery following removal of the upper green mat layer, when light was more available for deeper populations (Ferris et al. 1997). These observations suggest that distinct *Synechococcus* populations conduct photosynthesis at different depths, consistent with ecotype distribution results. As mentioned above, Ramsing et al. (2000) suggested that the vertical positioning of oxygenic photosynthesis (estimated from vertical oxygen profiles) moves to deeper mat layers as light intensity increases during the morning and this suggests that species residing at different depths in the photic zone are engaged in photosynthesis at different times of day. Spectral alteration of light that penetrates into the mat (Fig. 3.5b) may suggest adaptation or acclimation to light quantity and/or quality.

It was previously demonstrated using microsensors that (i) the *Synechococucs* spp. photosynthesize so intensely that their CO_2 consumption causes pH to rise from 8.4 in water flowing above the mat to ~9.4 (Revsbech and Ward 1984) and (ii) using $^{14}CO_2$ labeling, that these low CO_2/high O_2 microenvironmental conditions lead to photorespiratory production of glycolic acid, which may be transferred to other mat organisms (Bateson and Ward 1988). Other $^{14}CO_2$ labeling studies showed that mat *Synechococcus* populations produce mainly polyglucose as a consequence of oxygenic photoautotrophy during the day and shift their metabolism to polyglucose fermentation in the dark (Konopka 1992; Nold and Ward 1996), mainly to acetate, propionate and CO_2. Based on evidence of the photoassimilation of fermentation products by filamentous mat inhabitants (Sandbeck and Ward 1981; Anderson et al. 1987), it was hypothesized that *Synechococcus* spp. cross-feed fermentation products to *Roseiflexus* spp. and/or *Chloroflexus* spp. (Nold and Ward 1996) (Fig. 3.10a). This was demonstrated by stable isotope probing of diagnostic lipid biomarkers (van der Meer et al. 2005). In these experiments the mat was pulse-labeled with $^{13}CO_2$ during the afternoon, resulting in labeling of *Synechococcus* spp. lipids (and presumably polyglucose based on $^{14}CO_2$ labeling results mentioned above). After incubation during the night and into the following morning, lipids diagnostic of *Roseiflexus* spp. became labeled and the labeling could be blocked by increasing the pool size of unlabeled acetate and glycolate (Fig. 3.10b). Previous natural abundance stable isotope studies had demonstrated that *Roseiflexus* spp. lipid biomarkers were isotopically heavier than expected based on photoheterotrophic uptake of fermentation products derived from cyanobacteria, which use the Calvin-Bensen Cycle for fixing CO_2 (van der Meer et al. 2000, 2003). *Roseiflexus* sp. strain RS1, which is closely related to native *Roseiflexus* populations (van der Meer et al. 2010), was found to possess genes for photoautotrophy using the 3-hydroxypropionate cycle (Klatt et al. 2007). This pathway has a lower degree of isotopic fractionation than the Calvin-Bensen Cycle and could explain the heavier *Roseiflexus* spp. lipid biomarker signatures. van der Meer et al. (2007) showed the natural cycling of polyglucose in mat *Synechococcus* spp., which had been partially separated from other mat inhabitants using a Percol gradient (Fig. 3.11a). This separation enabled demonstration that the stable carbon isotopic fractionation in polyglucose biosynthesis is much lower than in lipid biosynthesis. Hence, the transfer of carbon, first fixed as polyglucose and then fermented by *Synechococcus* spp., coupled to the uptake of fermentation products by *Roseiflexus* spp. may also explain the heavy isotopic signatures in *Roseiflexus* spp. lipids. Recent evidence from diel metatranscription patterns, suggest that *Roseiflexus* spp. may use the 3-hydroxypropionate pathway in a mixotrophic, rather than an autotrophic process (Bryant et al. 2011; Klatt et al. 2012a).

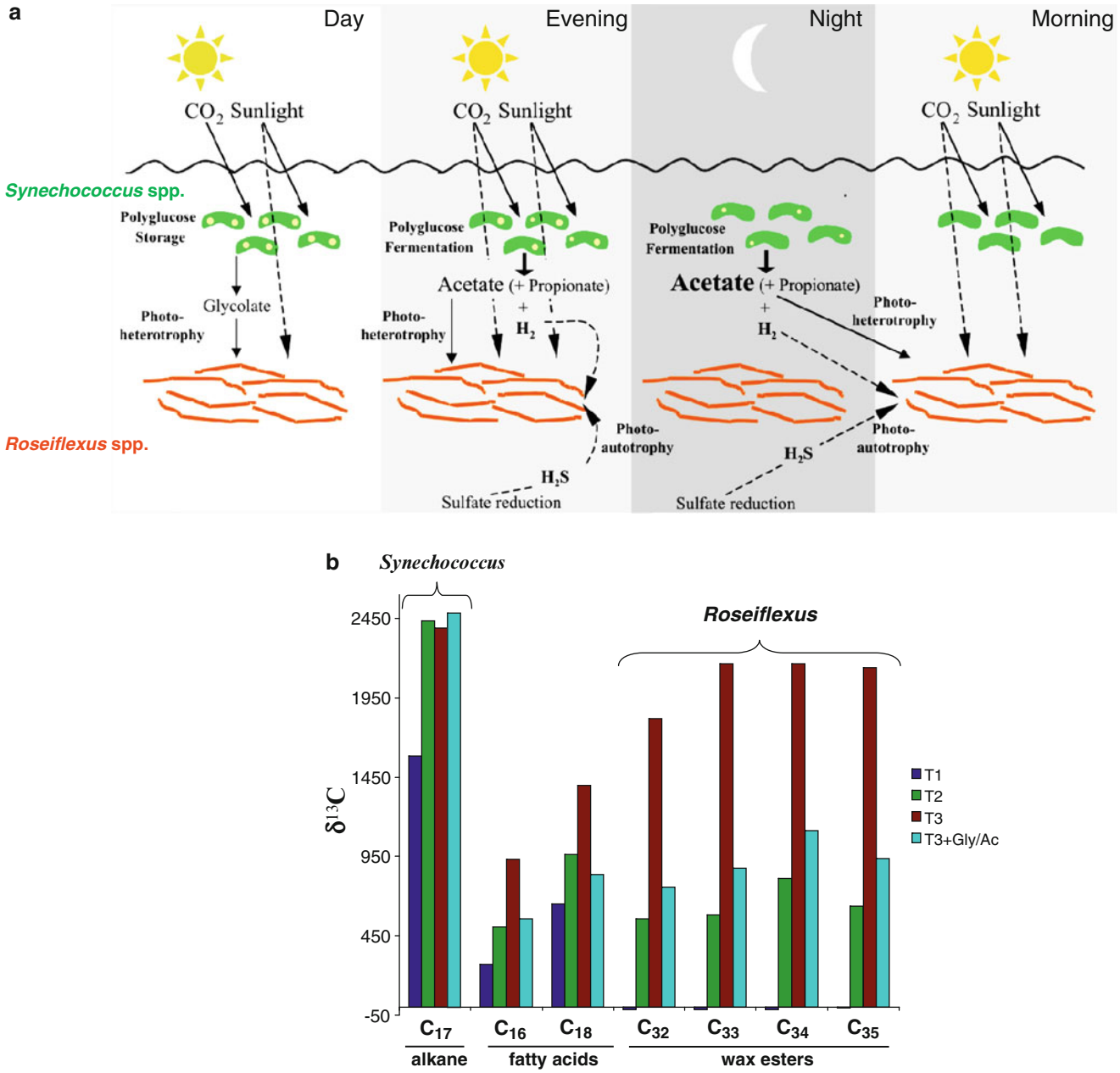
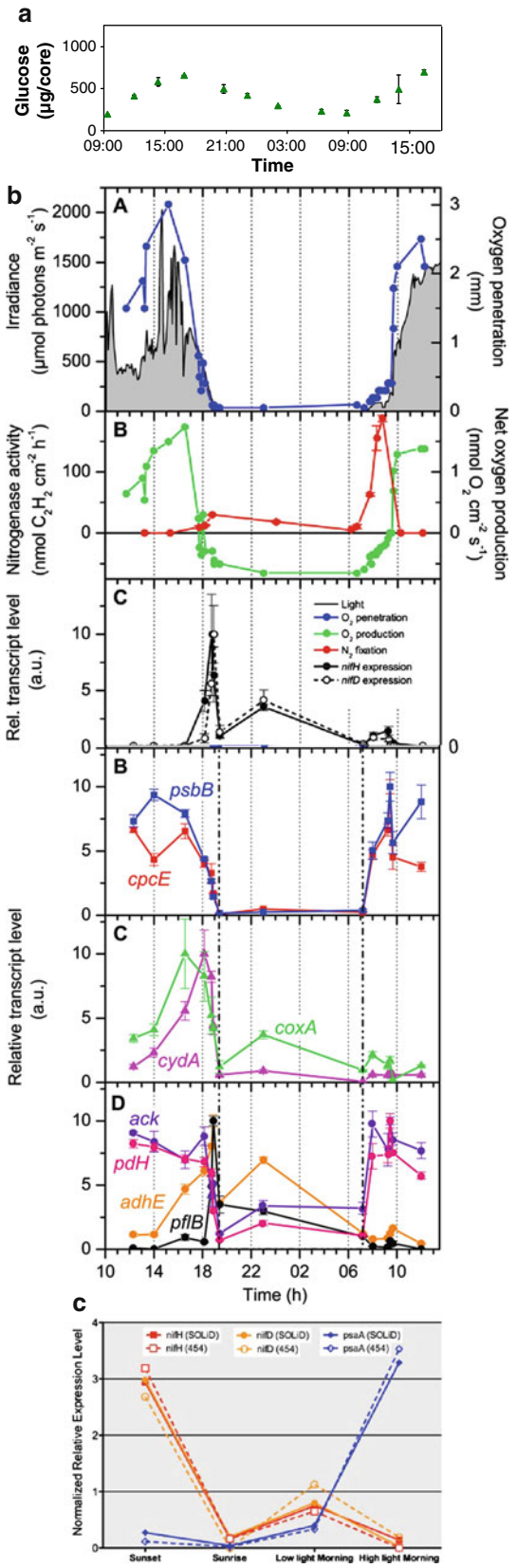


Fig. 3.10 **Cross feeding of metabolites from *Synechococcus* spp. to *Roseiflexus* spp.:** (**a**) Model postulating midday *Synechococcus* production of glycolic acid and polyglucose followed by a shift to fermentation at night and uptake of fermentation products in the morning by *Roseiflexus* spp. (**b**) Incorporation of $^{13}CO_2$ into diagnostic *Synechococcus* and *Roseiflexus* lipids in the afternoon (*T1*, 1,720 h), then, after removal of unmetabolized $^{13}CO_2$, redistribution of fixed ^{13}C resulting from continued incubation under natural light conditions until early morning (*T2*, 0645 h) and late morning (*T3*, 1055 h). Labeling between *T2* and *T3* was done with and without addition of a mixture of unlabeled glycolate and acetate at *T2* (*T3* + Gly/Ac) (Modified from van der Meer et al. 2005)

3.4.1.5 *In Situ* Gene Expression and Diel Metabolic Shifts

Steunou et al. (2006, 2008) used quantitative reverse transcriptase PCR to study the *in situ* transcription of B′-like *Synechococcus* genes in the ~60°C mat. These studies were focused on the transcription of genes associated with (i) nitrogen fixation, the genetic potential for which was discovered in the genomic analyses described above, and (ii) energy metabolisms that might drive this process throughout the diel light cycle. Interestingly, *nif* genes are transcribed in the evening (Fig. 3.11bC) when oxygenic photosynthesis no longer exceeds aerobic respiration and the mat becomes anoxic, except in the surface ~100 μm (Fig. 3.11bA). The upregulation of genes associated with aerobic respiration may indicate that *Synechococcus* spp. actively scavenge oxygen in the late afternoon (Fig. 3.11bE). Nif proteins are also produced and nitrogenase activity initiates (Fig. 3.11bB) in the evening. However, rates of N_2 fixation are low until morning, when a



burst of activity is observed. The low nighttime rates of N_2 fixation are presumably due to the low energy yield provided by polyglucose fermentation. The morning burst of N_2 fixation occurs when the mat receives diffuse light and remains anoxic because aerobic respiration exceeds photosynthetic oxygen production (Fig. 3.11bB). Once direct sunlight illuminates the mat, oxygen accumulates and *nif* gene transcripts and proteins and N_2 fixation, can no longer be detected. Genes involved in photosynthesis (Fig. 3.11bD) and some fermentation genes (Fig. 3.11bF) are expressed in a pattern consistent with the shift between these metabolisms in light and dark periods. Thus, it appears that *Synechococcus* spp. play a major role in the nitrogen economy and in recycling photosynthetically fixed carbon in the mat community. Jensen et al. (2010) demonstrated the *in situ* diel dynamics of the expression of B′-like *Synechococcus* genes associated with intense consumption of inorganic carbon (and resultant pH increase) and production of oxygen during oxygenic photosynthesis by such dense populations as occur in these mats. Transcripts of genes involved in both carbon concentration and reactive oxygen protection increased during the morning light transition and declined after the mid-day light, O_2 and pH maxima.

Metatranscriptomic analyses conducted using pyrosequencing and SOLiDTM sequencing confirm these targeted gene analyses (Fig. 3.11c), showing patterns of expression of N_2 fixation and photosynthesis genes that were nearly identical to those observed in the targeted analyses reported above (Liu et al. 2011). These global analyses will permit, in the very near future, detailed investigation of *in situ* physiology of *Synechococcus* spp. and other mat inhabitants. Resolution of A-like and B′-like *Synechococcus* transcription appears possible with such analyses (Liu et al. 2011). Targeted reverse-transcriptase PCR analysis of the *psaA* gene appears to permit analysis of species-specific transcription and these patterns are also seen in initial Ti454 metatranscriptomes (Becraft et al. 2012). Metatranscriptomic and metaproteomic analyses of samples collected at hourly intervals through a complete diel cycle are in progress (Liu et al. 2012 Ward, D.A. Bryant, L.Steinke,

Fig. 3.11 Diel metabolic rhythms in Mushroom Spring 60°C mat *Synechococcus* spp.: (**a**) Polyglucose levels associated with mat *Synechococcus* cells partially purified by Percol gradient separation (From van der Meer et al. 2007). (**b**) Light intensity and oxygen penetration (*A*), net oxygen production and nitrogenase activity (*B*), B′-like *Synechococcus* sp. gene transcripts involved in N_2 fixation (*C*), photosynthesis (*D*), aerobic respiration (*E*) and fermentation (*F*) (From Steunou et al. 2008) (**d**) Metatranscriptomic results for expression of N_2 fixaton (*nifH*, *nifD*) and photosynthesis (*psaA*) genes in samples collected at sunset, sunrise and at low-light and high-light morning periods (Data from Liu et al. 2011)

M.Lipton, unpublished data) and these should yield detailed information about the timing of gene expression and protein abundance. For any transcripts that offer as much molecular resolution as the *psaA* gene (and possibly some proteins), it may be possible to understand the functional ecology of *Synechococcus* spp. and other mat inhabitants at the species level.

3.4.2 White Creek *Fischerella*

F. laminosus is the predominant cyanobacterium at White Creek between approximately 39°C and 54°C mean annual temperature (Fig. 3.7). Recent efforts have provided insights into the distribution of genetic and ecological variation in this population (Miller et al. 2006, 2009b; Miller 2007, 2010) that complement work on thermal niche differentiation of the *Synechococcus* A/B group.

Although population members distributed along the White Creek thermal gradient exhibit no divergence at the 16S rRNA locus or the adjacent ITS region (Miller et al. 2006), they do harbor low amounts of genetic variation (~1 polymorphic site per kb) in other, less conserved, regions of the genome (Miller et al. 2006, 2009b). Extensive recombination observed among these variable markers (Miller et al. 2006, 2007, 2009b) acts to break up the genetic linkage that would otherwise result in genome-wide nonrandom associations between alleles at different loci. Most of this variation exhibits little or no distribution difference along the channel (Miller et al. 2009b; Miller 2010, unpublished data), which suggests a highly genetically-connected population that is not limited by dispersal. However, some variable markers (e.g. *nifH*, *rfbC*) show strong genetic differentiation (i.e. high F_{ST}) along the White Creek thermal gradient, with different alleles predominating at upstream and downstream zones, respectively (Miller et al. 2009b; unpublished data). A possible explanation for the above patterns of genetic polymorphism is that migratory gene flow is generally unrestricted in the population but is prevented in certain regions of the genome by natural selection.

Temperature is likely to be a selective agent that contributes to these barriers to gene flow, since genetically divergent strains of *F. laminosus* exhibit differences in thermal performance that closely match the prevailing conditions that they experience *in situ* (Miller et al. 2009b). Population members can therefore be considered to be ecological specialists that have diverged in their thermal niches. Crossing reaction norms between high temperature (55°C) and low temperature (37°C) performance among strains indicates that a trade-off at physiological extremes has likely contributed to niche differentiation of the population. Ongoing work seeks to identify the functionally important genetic variation that underlies these ecological differences.

3.5 Conclusions

Ward and Castenholz (2000) expressed the hope that the results produced by mentors studying cyanobacteria in geothermal habitats would, like those of the masons who envisioned the great cathedrals of Europe, but could not witness their completion, provide a solid framework for further subsequent independent research by their students and others. Happily, this has occurred, though not in exact terms.

An example of consistency can be found in patterns of temperature adaptation, which have been observed as new cyanobacterial isolates have been brought into culture, which are known to be genetically representative of the native populations from which they were obtained. Their temperature relations in culture (fundamental niche) have been shown to correlate with their *in situ* distributions (realized niche), and we are beginning to develop an understanding of how the evolution of physical properties of macromolecules have shaped observed differences in temperature relations. However, an example of inconsistency can be found in differences in the importance of particular types of cyanobacteria at particular temperatures in particular hot springs as revealed in high-throughput molecular analyses. For instance, *Synechococcus* genotypes have been seen to exhibit different temperature-defined realized niches in different hot spring effluents. Differences have also been noted in the predominant filamentous anoxygenic phototrophic populations in different hot spring effluent channels. These differences remind us that temperature is not the only determinant of niche. Indeed, genomic and metagenomic analyses have revealed nutritional differences suspected from earlier morphoytype distribution studies. These differences also remind us that the competitiveness of a population depends on all the various niche determinants. Thus, patterns observed in one system may not apply to another system.

A second example of consistency is that higher molecular resolution analyses have confirmed the inference made based on patterns seen in 16S rRNA distribution analyses that adaptation has been important in the evolution of geothermal cyanobacteria. In fact, when resolution is sufficient to view variation among individuals it is possible to conduct population genetics analyses that result in the demarcation of populations of ecologically interchangeable individuals equivalent to ecological species that have unique ecological adaptations. However, insufficient resolution may effectively lump these species populations into sets of populations that appear to be heterogeneous rather than homogeneous with respect to ecological uniqueness. Demarcation of taxa by morphological similarity is dangerous, as the number of species within a morphologically defined taxon can be very large and this can, in turn, obfuscate the

recognition of patterns suggesting adaptive and biogeographical differences. For instance, *Synechococcus* is genetically extremely diverse and exhibits different patterns of global distribution suggestive of divergence due to geographic isolation, some of which would have been masked by defining this genus on the basis of morphological similarity. *Fischerella*, while more genetically coherent, nevertheless also exhibits population structure that indicates that adaptation to temperature and physical isolation are associated with genetic divergence. These observations confirm the generalizations of Castenholz (1992) that "Obviously, cyanobacterial species are the results of natural selection … Also, some species have very restricted geographic distributions, suggesting that allopatric speciation can be important in cyanobacterial evolution." But, his observation that "most species or strains of cyanobacteria seem to have been selected for a wide tolerance of environmental extremes or simply for a great 'ecological amplitude'" may need to be reinvestigated at higher resolution, if based on morphotype distribution patterns.

Well-studied hot spring microbial mat systems continue to serve as excellent models from which to make discoveries of general importance to microbial community ecology. Their extremeness provides relative simplicity, which makes them highly tractable for study by metagenomic, metatranscriptomic and metaproteomic techniques. These methods offer the advantage of being global with respect to their ability to address comprehensively the question of "who is there" in a microbial community. Metagenomic assembly analyses confirm the observations made from more targeted genetic analyses of the predominant cyanobacterial taxa, but have also enabled the discovery of many novel and previously unsuspected noncyanobacterial community members, with whom the cyanobacteria interact. In essence, these "omics" methods have enabled a more objective analysis of a community system, freeing the microbial ecologist from unforeseen inadequacies of traditional approaches and ways of thinking about microbial communities. Metagenomics also provides a means of linking the phylogenetically defined populations to their potential function. Similarly, metatranscriptomics and metaproteomics are beginning to provide global analyses aiming in the direction of community function ("who is transcribing and translating what genes, when and where?"), possibly even to the species level of resolution. When combined with the kinds of labeling experiments that are possible in these relatively simple systems, it may even be possible to associate actual functions with the species responsible. In the future, we expect these systems to be excellent models for understanding the networking among species in the various functional guilds of the community, hopefully providing insights that will be important for microbial systems in more moderate environments that are not so tractable for "omics" analyses.

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