



# Cyanidiales diversity in Yellowstone National Park

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# Cyanidiales diversity in Yellowstone National Park

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**Significance and Impact of the Study:** The Cyanidiales are an order of unicellular eukaryotic algae that thrive in acidic geothermal environments. In this study, we report several novel observations relative to Cyanidiales ecology in Yellowstone National Park, including the following: (i) the identification of two phylogenetic lineages of Cyanidiales: Cyanidioschyzon and Galdieria; (ii) the absence of Galdieria in aquatic environments; (iii) the absence of Cyanidium and Galdieria phlegrea in prime Cyanidiales habitats; (iv) the cohabitation of Cyanidioschyzon and Galdieria in nonaqueous environments; and (v) the first in situ evidence regarding the relationship between soil moisture and Cyanidiales habitat preference and viability.

## Abstract

The Cyanidiales are unicellular red algae that are unique among phototrophs. They thrive in acidic, moderately high-temperature habitats typically associated with geothermally active regions, although much remains to be learned about their distribution and diversity within such extreme environments. We focused on Yellowstone National Park (YNP), using culture-dependent efforts in combination with a park-wide environmental polymerase chain reaction (PCR) survey to examine Cyanidiales diversity and distribution in aqueous (i.e. submerged), soil and endolithic environments. Phylogenetic reconstruction of Cyanidiales biodiversity demonstrated the presence of Cyanidioschyzon and Galdieria lineages exhibiting distinct habitat preferences. Cyanidioschyzon was the only phylotype detected in aqueous environments, but was also prominent in moist soil and endolithic habitats, environments where this genus was thought to be scarce. Galdieria was found in soil and endolithic samples, but absent in aqueous habitats. Interestingly, Cyanidium could not be found in the surveys, suggesting this genus may be absent or rare in YNP. Direct microscopic counts and viable counts from soil samples collected along a moisture gradient were positively correlated with moisture content, providing the first in situ evidence that gravimetric moisture is an important environmental parameter controlling distribution of these algae.

## Keywords

algae, diversity, ecology, environmental, microbial physiology.

## Introduction

The thermo-acidophilic, unicellular red algae of the order Cyanidiales are unique in that their environment is defined by acidic pH (0–5) and elevated temperature (38–56°C). They are a dominant component of microbial communities inhabiting acidic geothermal environments, being present in springs, soils and endolithic habitats throughout the world (Brock 1978; Seckbach 1994, 1999; Castenholz and McDermott 2010; Reeb and Bhattacharya 2010). Cyanidiales taxonomy was originally based on morphology, with their simple cell morphology leading to the identification of only three genera: Cyanidium, Cyanidioschyzon and Galdieria (Gross et al. 2001; Heilmann and Gross 2001; Seckbach 2010). It has been suggested that the limited morphological variation has led to

underestimating their genetic diversity, with many novel species remaining to be elucidated (Ciniglia *et al.* 2004). These, along with many aspects of cyanidial ecology, remain poorly explored and represent high potential for new discoveries with regard to Cyanidiales distribution and diversity.

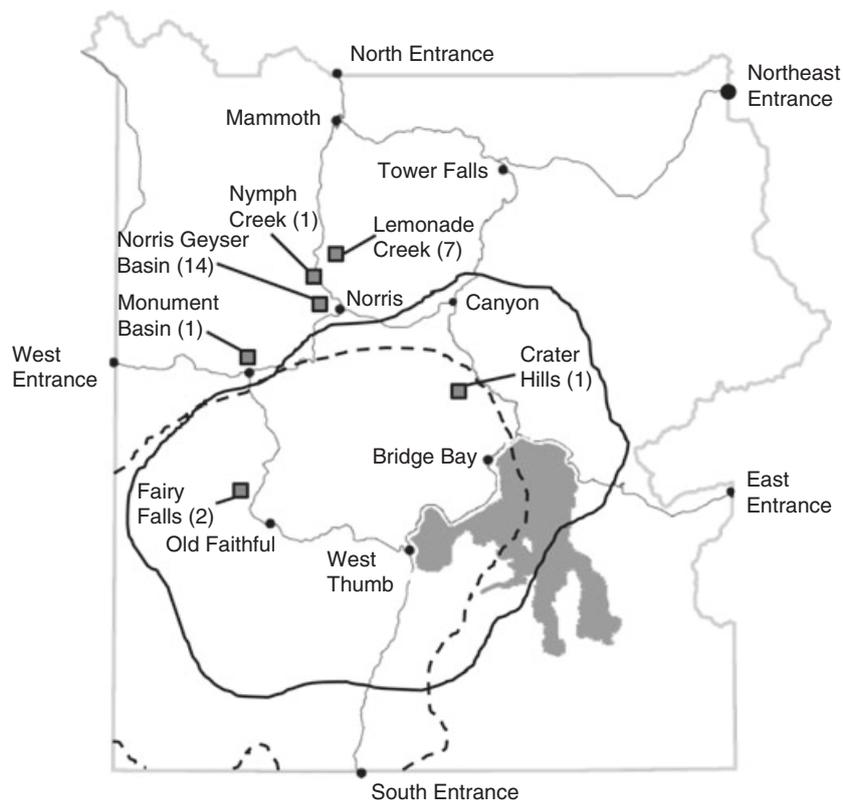
A renowned geothermal environment is the Yellowstone National Park (YNP) geothermal complex, a c. 9000 km<sup>2</sup> region with over 11 000 hydrothermal features that vary widely in pH, temperature and chemistry (Nordstrom *et al.* 2005). Various environmental factors could exert selection pressures on the Cyanidiales, potentially resulting in cyanidial distribution patterns within YNP. Doemel and Brock (1971) suggested moisture may be important, however, limited information is available regarding Cyanidiales desiccation (in) tolerance and species distribution. Cyanidiales isolates have been examined for their response to dehydration (Pinto *et al.* 2007), although it is unknown how pure culture observations relate to *in situ*. In the current study, cultivation efforts and molecular surveys were used to examine Cyanidiales in YNP soil, endolithic and aqueous habitats. Our goals were to: (i) assess Cyanidiales diversity and ecology using culture-independent approaches; (ii) attempt to identify *Cyanidium*, a genus thus far undetected in YNP; and (iii) examine

and quantify the ecological relationship of these algae with moisture *in situ*.

## Results and discussion

### Molecular survey of Cyanidiales diversity and distribution

Six geographically dispersed geothermal areas within YNP that varied with respect to temperature, pH, moisture content and/or habitat type were sampled at multiple locations for the Cyanidiales diversity survey (Fig. 1). Descriptions of these sites are provided in Table S1, with some representative images of habitat types shown in Fig. S1. Several geothermal areas were more intensely sampled because they offered a variety of contrasting environments or thermal gradients (e.g. Fig. S1A) and were in close proximity to each other (cm to <m) to limit migration barriers. This included submerged mats with temperature gradients (Fig. S1C) that were directly (1–2 cm) adjacent to endolithic-like environments (Fig. S1B) that were small ledges (Fig. S1D) adjacent (<1 m) to sampled soils (Fig. S1E, F). Concerted sampling at these locations aimed to fully capture the species composition, reducing the possibility of missing minor components of community composition. A total of 282 *rbcl*-encoding clones were



**Figure 1** Map of Yellowstone National Park (YNP) and approximate locations of the sampling sites described in this study. Large grey squares indicate geothermal areas sampled for Cyanidiales *rbcl* diversity: Fairy Falls trail head (FF); Monument Basin (MB); Norris Geyser Basin (NGB); Nymph Creek (NC); Lemonade Creek (LC); and Crater Hills (CH). Specific geothermal area information is described in detail in Table S1. The number of sites sampled at each location is shown in parentheses. Solid and dashed black lines represent the approximate rim of the 0-64 and 2 my YNP caldera boundaries.

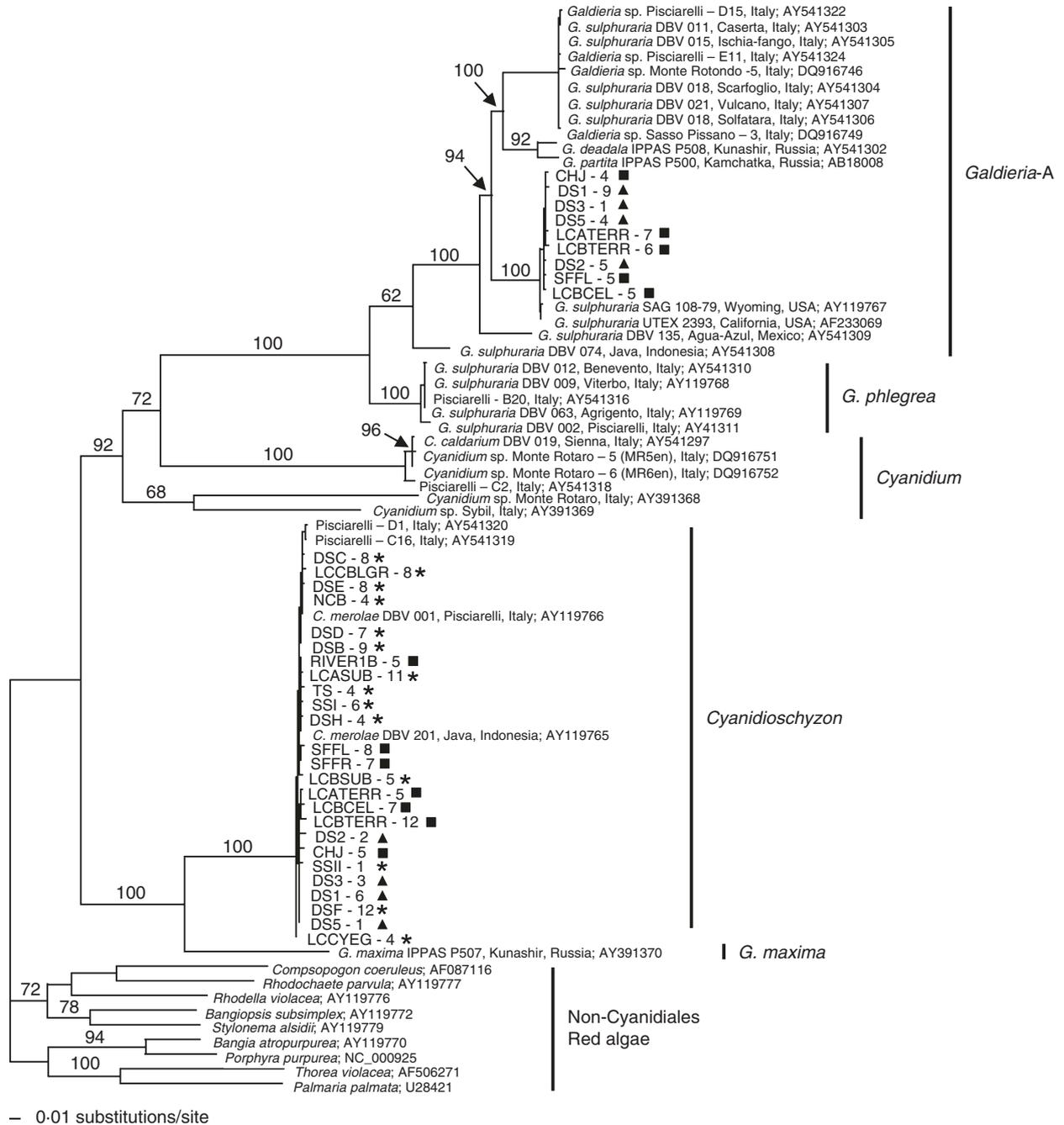
sequenced from 25 different sampling sites. All sites met the most common Cyanidiales habitat requirement (elevated temperature and acidic pH). This molecular approach to investigating Cyanidiales diversity in YNP was employed as a complementary study to Toplin *et al.* (2008), which was entirely cultivation dependent. It is well known that cultivation-based studies are biased with respect to characterizing the relative diversity of microorganisms present, and thus it was important to conduct a follow-up analysis that would avoid such biases and allow for more intense sampling and more comprehensive diversity estimates than could be derived from fewer individual isolates.

Sequence diversity at each site was very low (maximum nucleotide divergence = 0.5%) with no evidence of clone variation or diversity across gradients. Consequently, a single representative *rbcL* clone from each cyanidial species at each site was selected for near full-length (1215 nt) sequencing and phylogenetic analysis. From a total of 34 full-length clones, Cyanidiales *rbcL* diversity throughout the Yellowstone geothermal complex resolved into two major YNP phylotypes among all currently identified Cyanidiales lineages; *Galdieria*-A [clade designation following Ciniglia *et al.* (2004)] and *Cyanidioschyzon*, with strong bootstrap support (Fig. 2). Members of the largest group of clones, identified as *Cyanidioschyzon*, were 99.3% identical to the *rbcL* of the fully sequenced *Cyanidioschyzon merolae* strain 10D genome. *Cyanidioschyzon* sequences were found in high proportional occurrence in endolithic and soil environments, comprising 42–100% of the total clone abundance in endolithic environments and 10–55% in sampled soils (Table S1). The widespread co-occurrence, and in some cases apparent dominance of *Cyanidioschyzon* populations in nonaqueous environments, expands on the distribution of this genera, which was previously thought to be only a minor component of the algal composition such habitats (Pinto *et al.* 2007). *Cyanidioschyzon* was also the only lineage identified among the 170 *rbcL* clones derived from submerged mats (Fig. 2, Table S1). The collective data suggest that the ecological plasticity of YNP *Cyanidioschyzon* is greater than that of *Galdieria*, which was encountered less frequently in the various *rbcL* libraries and never in aqueous environments (Fig. 2, Table S1). Such plasticity likely plays a role in enhancing geographic dispersal. Isolates from Indonesia and Italy are phylogenetically closely related to the YNP *Cyanidioschyzon* populations (Fig. 2). We note that *Galdieria daedala*, *Galdieria maxima* and *Galdieria partita* failed to demonstrate the same clade structure as reported by Cozzolino *et al.* (2000) using a much smaller selection of *rbcL* sequences.

The other major lineage, *Galdieria*-A, was found in soil and endolithic habitats but never in aqueous samples and

grouped together with *Galdieria sulphuraria* isolates from YNP and Sonoma, CA, but was distinct from *Galdieria*-A clones from Italy, Russia and Mexico (Fig. 2). It was also different from a *Galdieria phlegrea* clade [referred to as *Galdieria*-B, see Pinto *et al.* 2007] comprised of cultures isolated from geothermal environments in Italy (Fig. 2). The *rbcL* clone sequences from this study firmly establish what was previously only a putative North American *Galdieria*-A subclade (Fig. 2), now forming a robust and distinct North American lineage. However, the subclade did not include the *G. sulphuraria* DBV 135 isolate from Mexico (Fig. 2), strengthening the argument that dispersal events for these algae may be extremely infrequent, and likely due to some Cyanidiales species having a low tolerance to desiccation and mesophilic conditions (Castenholz and McDermott 2010). Irregular dispersal events would create isolated populations that, given sufficient time, could genetically differentiate to form geographically distinct clades (Miller *et al.* 2007; Yoon *et al.* 2010). The absence of *Galdieria* from submerged mats contrasts the facile detection of this genus in humid environments in the Pisciarelli (Naples, Italy) hydrothermal region (Ciniglia *et al.* 2004). It is possible that *Galdieria* phylotypes are present in low abundance in humid habitats in YNP, however, their abundance would be <0.7% based on current sampling and sequencing efforts.

*Cyanidium* was not encountered in any of the clone libraries. Additional PCRs were conducted using *Cyanidium*-specific *rbcL* primers with the same DNA extracts from all sampling locations, including sites that were heavily sampled (e.g. 111 clones from Dragon Spring and 80 clones from Lemonade Creek sampling sites). All PCRs were negative, which is in stark contrast to the abundance of *Cyanidium* in small clone sets (17–82% of clones sequenced) obtained from the Sasso Pisano and Monte Rotondo geothermal locations in the Larderello region of Tuscany (Yoon *et al.* 2006) and Pisciarelli, Italy geothermal fields (Ciniglia *et al.* 2004). Failure to detect *Cyanidium* with PCR primers known to detect this taxon (and that consistently amplified positive controls) serves to offer nonbiased confirmation of the Toplin *et al.* (2008) study that also failed to identify *Cyanidium* in a very large culture collection. These combined studies surprisingly suggest this genus is either absent in YNP or such a minor component of the YNP Cyanidiales that it is difficult to detect with even molecular approaches. Sequencing results with the general Cyanidiales primers lead us to conclude that if present, *Cyanidium* phylotypes may exist at abundance levels no greater than 0.35%, a low estimate that is consistent with the absence of a PCR amplicon with *Cyanidium*-specific primers, although the latter experiments argue this organism is simply absent. Alternatively, the genus may exist in refugia yet to be sampled,



although we note that the sites sampled represent prime Cyanidiales habitat from across the YNP complex. It has been suggested that past major volcanic events may have shaped contemporary microbial biogeographical patterns in YNP (Takacs-Vesbach *et al.* 2008); however, Cyanidi-

ales phylotypes recovered from the Crater Hill and Fairy Falls sampling sites (inside both the 0-640 and 2 million year caldera boundaries, Fig. 1) did not differ from Cyanidiales habitats outside the caldera boundaries. This suggests that past volcanic events did not have a detectable

impact on these taxa or that an extinction event was followed by recolonization by taxa that did not include *Cyanidium*. We conclude that either the YNP geothermal environments differ in a fundamental way so as to exclude or be far less accommodating to *Cyanidium*, or YNP's past geologic events may have somehow selectively altered the ecology of this taxon.

### Cultivation experiments

Whereas the molecular surveys indicated that *Galdieria* was absent in aqueous environments, both *Cyanidioschyzon* and *Galdieria* phylotypes were present in nonaqueous habitats. Moisture is viewed as an important ecological factor underlying Cyanidiales niche separation (Ciniglia *et al.* 2004; Yoon *et al.* 2006; Pinto *et al.* 2007), although it has never been examined *in situ*. Thus, sampling efforts in subsequent cultivation studies focused on environments that varied in moisture levels. The Dragon Spring sampling site contained two distinct Cyanidiales habitats in close proximity to each other (soil and aqueous outflow channel within *c.* 0.8 m of each other) and thus avoided geographical barriers to dispersion and allowing for natural cross-colonization. Soils adjacent to Dragon Spring (Table 1) become progressively drier with increasing distance from the edge of the spring outflow channel (which was colonized exclusively by *Cyanidioschyzon*, sites DSB-DSI, Table S1), establishing an *in situ* sampling transect with a moisture gradient within less than a metre of an aqueous environment. This allowed us to investigate the habitat separation that appears to prevail between the YNP *Galdieria* and *Cyanidioschyzon* in the absence of long distance geographical dispersion barriers. All sampled

habitats were acidic (pH 2.1–2.7), with gravimetric moisture contents ranging from 8.1–30.8%. *Galdieria-A* was the only alga observed among 120 PCR clones generated from these soil DNA samples (Table 1).

In addition to the molecular analysis, the effect of moisture was determined using direct microscopic counts and viable plate counts (both autotrophic and heterotrophic). Table 1 provides results from one of three experiments illustrating similar soil moisture influences. Both types of counts were significantly positively correlated with gravimetric soil moisture content: microscopic counts ( $n = 32$ ),  $r^2 = 0.965$ ,  $P$ -value  $< 0.01$ ; viable counts ( $n = 12$  for each autotrophic and heterotrophic count at each level of soil moisture),  $r^2 = 0.977$ ,  $P$ -value  $< 0.01$ . An analysis of variance and Duncan's multiple range test determined that for total microscopic counts, with exception of the 30.1 and 30.8% moisture soils, all means were significantly different ( $P$ -value  $< 0.05$ ). For viable plate counts (both autotrophic and heterotrophic), mean differences were not significant when comparing the 30.8 and 30.1% soil moisture samples, nor were they different when comparing the 18.5 and 8.1% soil moisture samples. However, the order of magnitude decrease in viability when soil moisture dropped from 30.1 to 18.5% was significant ( $P$ -value  $< 0.05$ ), suggesting a critical moisture threshold had been reached. These results obtained with *in situ* samples as opposed to moisture manipulation in laboratory experiments firmly establish that moisture is a critical environmental parameter controlling Cyanidiales assemblages and viability. Autotrophic and heterotrophic colony counts were not statistically different (Table 1), and because heterotrophy is a physiological feature that is exclusive to *Galdieria*, this suggested these soils were colo-

**Table 1** Cyanidiales microscopic and viable counts along a sampling transect across increasingly drier soils. Viable plate counts were obtained on Allen's agar without (autotrophic) or with (heterotrophic, incubated in the dark) mannose added as a carbon source. Estimates of Cyanidiales *rbcl* clone composition at each transect position are also included

Transect position (m)*	pH	Soil moisture content (%)	Counts (10 <sup>5</sup> cells/g-soil)			Cyanidiales composition (%)†	
			Total microscopic	Viable		<i>Cyanidioschyzon</i>	<i>Galdieria</i>
				Autotrophic	Heterotrophic		
0.8	2.7	30.8	8.8 ± 1.5a§	2.02 ± 0.380a (23.0)‡	2.09 ± 0.050a (23.8)‡		100
0.8	2.6	30.1	9.2 ± 1.6a	1.88 ± 0.010a (20.4)	1.67 ± 0.310a (18.2)		100
4.9	2.1	18.5	5.7 ± 1.1b	0.10 ± 0.009b (1.8)	0.10 ± 0.009b (1.8)		100
6.4	2.7	8.1	1.4 ± 0.2c	0.02 ± 0.002b (1.4)	0.04 ± 0.002b (2.9)		100

\*Distance from the edge of the Dragon Spring outflow channel.

†A total of 30 PCR amplified *rbcl* clones were sequenced for each soil site.

‡Mean ± standard deviation of triplicate plate counts, with the proportion of total counts (%) shown in parentheses. Means followed by the same letter do not differ significantly from one another ( $P = 0.05$ ).

§Mean ± standard deviation of triplicate microscopic counts. Means followed by the same letter do not differ significantly from one another ( $P = 0.05$ ).

nized primarily by *Galdieria*. This was confirmed by the colony phylotyping (30 colonies each for autotrophic and heterotrophic plates for each soil, 240 colonies), which together with PCR cloning and sequencing from soil DNA suggested that all colonies were *Galdieria* (Table 1). The absence of *Cyanidioschyzon* in the soil transect likely derives from the fact that during the summer months, when samples were collected, these soils dry too extensively to maintain an alga that appears to require significant moisture in its immediate environment.

In summary, this study documented several novel findings with respect to Cyanidiales ecology and evolution: (i) the identification of two major *rbcL* phylogenetic lineages of Cyanidiales in YNP; (ii) the absence of *Galdieria* in aquatic environments; (iii) the apparent absence of *Cyanidium* and the *Galdieria phlegrea* lineages in prime Cyanidiales habitats; (iv) the cohabitation of *Cyanidioschyzon* and *Galdieria* in soil and endolithic environments; and (v) the first *in situ* evidence regarding the relationship between soil moisture and Cyanidiales habitat preference and viability. Clearly, liquid water is a major environmental feature controlling cyanidial distribution and abundance.

## Materials and methods

### Site descriptions and sample collection

Samples were taken from various thermo-acidic areas within YNP (WY, USA) (Figs 1 and S1, Table S1), and within certain sites sampling targeted temperature gradients. Soil materials were collected using sterile stainless-steel spatulas, whereas autoclaved large bore pipet tips were used for acquiring aqueous mat samples (defined as submerged in hot spring outflow channels). For endolithic samples, a sterile hammer was used to break off the upper rock layer to expose the underlying endolithic algae, which was then scraped and collected using an autoclaved spatula. Samples were placed in sterile 50 ml Falcon™ tubes, flash frozen in a dry ice/ethanol bath and stored at  $-80^{\circ}\text{C}$  until DNA extraction.

### DNA extraction, *rbcL* cloning, sequencing and phylogenetic analysis

DNA was extracted from all samples as described previously (Botero *et al.* 2004). The nearly full-length *rbcL* gene was amplified using the protocols and the Cyanidiales-specific primers *rbcL*-90F and *rCR* as described previously [(Yoon *et al.* 2002; Ciniglia *et al.* 2004) Table S2]. Amplicons were purified (QIAquick PCR purification kit, Qiagen, Valencia, CA, USA) and cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA). From

each of the 25 environmental samples, 10–12 clones (282 total) were sequence screened to identify clones of interest for full-length sequencing (High-Throughput Sequencing Solutions, Seattle, WA, USA; Nevada Genomics Center, Reno, NV, USA; Molecular Research Core Facility, Pocatello, ID, USA). Sequences were edited using Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned using ClustalX. Phylogenetic analysis of aligned YNP clones and reference sequences used maximum likelihood analysis with the PAUP\* software package (Swofford 2002). Bootstrap values were generated using 100 pseudoreplicates in a heuristic search (starting with a random tree), and the tree bisection-reconnection branch-swapping algorithm was used to find the best tree. Clone sequences described in this study can be found as GenBank accessions JQ269605–JQ269638.

### *Cyanidium*-specific PCR primers and amplification

*Cyanidium* was specifically targeted with the universal Cyanidiales forward primer *rbcL*-90F (Ciniglia *et al.* 2004) paired with three different reverse primers (MR-*rbcLR*, DBV182-*rbcLR* or Sybil-*rbcLR*) designed from the three *Cyanidium rbcL* group sequences (Table S2). PCRs were as described above with template DNA obtained from each of the 25 survey sites. Annealing temperatures were  $51^{\circ}\text{C}$  for MR-*rbcLR*,  $55^{\circ}\text{C}$  for DBV182-*rbcLR* and  $45^{\circ}\text{C}$  for Sybil-*rbcLR*. Positive controls from each of the three *Cyanidium* groups were included to ensure both primer specificity and positive amplification.

### Cultivation experiments

Soil sites near Dragon Spring in Norris Geyser Basin were sampled and used to estimate the proportional occurrence of cyanidial populations in nonaqueous habitats. Sample material was aseptically collected as described above, but without freezing and transferred to the laboratory. Samples were serially diluted in Allen's medium (Allen 1959), with aliquots plated in triplicate onto solid Allen's medium ( $20\text{ g l}^{-1}$  Gelrite™, Research Products International Corp., Mt. Prospect, IL, USA) at pH 3.5. Two sets of solid media were inoculated as follows: Allen's for autotrophic (i.e. total phototrophs) counts and Allen's amended with  $10\text{ mmol l}^{-1}$  mannose to support heterotrophic growth (Gross *et al.* 1998; Oesterhelt *et al.* 2007). Autotrophic plates were incubated at  $37^{\circ}\text{C}$  under constant illumination from cool-white fluorescent lamps ( $80\text{ }\mu\text{E m}^{-2}\text{ s}^{-1}$ ), whereas heterotrophic Allen's medium plates were incubated in continuous darkness at  $37^{\circ}\text{C}$ .

Viable cell counts were based on colony-forming units (30–300/plate). Direct counts were performed using a

Zeiss Axioskop 2 microscope (Zeiss, Jena, Germany) and a Petroff-Hauser counting chamber (Hausser Scientific, Horsham, PA, USA). All counts were normalized based on dry weight of the soil or rock material, which was determined by drying overnight at 65°C and then weighing to determine gravimetric moisture content. Nonlinear regression analysis (one-variable model, R software version 2.12.1, <http://www.r-project.org>) was used to examine statistically significant correlations between cell counts and soil moisture. Statistical comparison between mean microscopic and viable cell counts along the sampling transect was conducted using a one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (MRT). Species composition for all soils was determined using culture-independent molecular methods, using environmental DNA extraction, *rbcL* amplification and cloning methods as described above. For each soil sample, the *rbcL* phylotype was also determined for 30 colonies arising on the autotrophic and heterotrophic plates.

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## Conflicts of interest

No conflict of interest declared

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Images of Lemonade Creek, one of the sampling areas in this study.

**Table S1.** Sampling site locations, habitat type, physical features, and estimates of Cyanidiales composition.

**Table S2.** *rbcL* PCR primers used in this study.