Extremophiles (2009) 13:707–716
DOI 10.1007/s00792-009-0258-y

ORIGINAL PAPER

An unusual cyanobacterium from saline thermal waters with relatives from unexpected habitats

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Received: 22 August 2008 / Accepted: 3 June 2009 / Published online: 20 June 2009

Abstract Cyanobacteria that grow above seawater salinity at temperatures above 45°C have rarely been studied. Cyanobacteria of this type of thermo-halophilic extremophile were isolated from siliceous crusts at 40–45°C in a geothermal seawater lagoon in southwest Iceland. *Iceland Clone 2e*, a *Leptolyngbya* morphotype, was selected for further study. This culture grew only at 45–50°C, in medium ranging from 28 to 94 g L^{-1} TDS, It showed 3 doublings 24 h^{-1} under continuous illumination. This rate at 54°C was somewhat reduced, and death occurred at 58°C. A comparison of the 16S rDNA sequence with all others in the NCBI database revealed 2 related *Leptolyngbya* isolates from a Greenland hot spring (13–16 g L^{-1} TDS). Three other similar sequences were from *Leptolyngbya* isolates from dry, endolithic habitats in Yellowstone National Park. All 6 formed a phylogenetic clade, suggesting common ancestry. These strains shared many similarities to *Iceland Clone 2e* with respect to temperature and salinity ranges and optima. Two endolithic *Leptolyngbya* isolates, grown previously at 23°C in freshwater medium, grew well at 50°C but only in saline medium. This study shows that limited genotypic similarity may reveal some salient phenotypic similarities, even when the related cyanobacteria are from vastly different and remote habitats.

Keywords Cyanobacteria · *Leptolyngbya* · Thermophile · Halophile · Endolith · Iceland · Greenland · Yellowstone

Abbreviations

bp	Before present
TDS	Total dissolved solids
SE	Standard error
CCMEE	Culture collection of microorganisms from extreme environments

Introduction

Numerous cyanobacteria are thermophiles (Ward and Castenholz 2000) and many are known as halophiles, even as extreme halophiles (Oren 2000), but there is little information on cyanobacteria that combine these 2 traits. Abed et al. (2002), however, characterized (by 16S rDNA sequences) a few very thin *Leptolyngbya*-like cyanobacteria (~1 μm diameter) that grew up to salinities somewhat above 120 g L^{-1} TDS, one of which grew slowly at a temperature as high as 50°C. These were isolated from a microbial mat in an artificial solar pond near Eilat, Israel and were named *Halomicronema excentricum* (Abed et al. 2002).

Another cyanobacterium, known for its great metabolic versatility, sometimes occurred in the solar-heated Solar Lake, Egypt, which was known seasonally to reach temperatures of about 60°C in the monimolimnion (see Oren 2000; Cohen et al. 1977). However, in culture this isolate, then known as "*Oscillatoria limnetica*" [renamed *Leptolyngbya hypolimnetica* (Campbell) Anagnostidis] was grown at

Communicated by F.Robb.

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>174 g L^{-1} TDS, but only at 35°C. However, growth has been demonstrated at 45°C (see Oren 2000). Little phenotypic information has been collected for this cyanobacterium with respect to salinity and temperature tolerance and optimum (Cohen et al. 1975). Another halophilic cyanobacterium, *Dactylococcopsis salina* from Solar Lake, has also shown growth at 45°C (see Javor 1989). Extremely halophilic *Aphanothece* spp. (=*Halothece*) from a solar pond near the Dead Sea grew at 48°C but not at 50°C (Dor and Hornoff 1985). Thermo-halophilic cyanobacteria may be expected in many areas of warm saline waters, such as salt evaporation ponds, or intertidal flats, especially in hot dry regions. However, such physiological types have seldom been isolated and characterized. In saline geothermal springs or ponds, such as the present site in Iceland, similar cyanobacteria may also be expected, but these habitats are very rare.

In the current study 5 *Leptolyngbya*-like cyanobacteria were isolated from the warm to hot saline waters of the Blue Lagoon (Bláa Lónid) on the Reykjanes Peninsula (southwestern Iceland) (Fig. 1). The altered seawater of the Blue Lagoon source water originates from a very hot terrestrial "outflow" of the mid-Atlantic Ridge and is used by a geothermal power plant. The water is then wasted and emptied into the lagoon after cooling to 40–50°C. A cyanobacterial band occurred in the siliceous crust along the upper submerged edge of the lagoon (Fig. 1).

The objectives of the present study were to determine the physiological characteristics (temperature and salinity optima) of the Iceland *Leptolyngbya*-like isolate and search for any related strains (based on 16S rDNA sequence close similarities). Five other *Leptolyngbya*-like culture isolates were found from the NCBI database. These were from different habitats and geographic areas and were compared to the Iceland strain with respect to growth characteristics in different combinations of temperature and salinity, desiccation and freezing tolerances, and also with respect to morphology.

Fig. 1 A view of the Blue Lagoon. This bathing area varied in temperature from ~40 to >45°C, the latter at a more distant point. The *bluish color* is due to the refraction and reflection of the heavy load of silica in suspension. The cyanobacteria were located in the narrow white area at the water–rock interface (see *arrow*)

Materials and methods

Sampling, isolation and culture methods

Collections were made in August, 2006 from scrapings of the rocky, siliceous edge of the saline Blue Lagoon (Bláa Lónid) which was bathed by water at 40–45°C (Fig. 1). These scrapings were plated centrally on IO BG-11 plates [25 g L^{-1} "Instant Ocean"™ (Aquarium Systems, East Lake, OH, USA) = ~28 g L^{-1} TDS + 1.8 g L^{-1} agar] after transport at room temperature in the dark from Iceland to the University of Oregon laboratory (see Castenholz 1988a, b). The filaments (trichomes) were allowed to both migrate and grow out from the central source of inoculum. Minute agar blocks with an individual self-isolated trichome (clone) were removed with a sterile watchmaker's forceps under 40–60× magnification with a dissecting microscope, and transferred into 125 mL capacity flasks of liquid IO BG-11 medium. After successful growth in flasks, the cultures were re-plated for a greater assurance of clonality. Five clonal cultures were then established and labeled *Iceland Clone 2*, a,b,c,d, and e. Since all were identical morphologically and with respect to 16S rDNA sequences, only one was chosen for the physiological experiments. All cultures were determined to be axenic, based on 100× Nomarski examination for contaminating heterotrophic bacteria and by plating on agar medium containing 90% IO BG-11 + dilute (10%) Plate Count Agar (Difco, Detroit, Michigan) that contains tryptone (5 g L^{-1}), yeast extract (2.5 g L^{-1}) and glucose (1 g L^{-1}). The isolates were identified as morphotypes fitting the description of the "form-genus" *Leptolyngbya* (Castenholz et al. 2001). These and cultures from other sources were grown in BG-11 or IO BG-11 medium (Castenholz 1988a, b), the latter with additions of NaCl as needed. Cultures of *Iceland Clone 2e* were maintained at 45 ± 1°C, and illuminated continuously with coolwhite fluorescent lamps (Sylvania, Danvers, MA, USA) under a photon flux of ~150 μE $m^{-2}s^{-1}$.

Geographic sources of cultures and the growth conditions

In addition to *Iceland Clone 2e*, other *Leptolyngbya*-like strains from endolithic habitats in Yellowstone National

Park showed 16S alignment with this cyanobacterium. These are *Leptolyngbya* spp. in the culture collection of microorganisms from extreme environments (CCMEE) 6132; *Leptolyngbya* sp. CCMEE 6111, and *Leptolyngbya* sp. CCMEE 6116 (Norris and Castenholz 2006). These strains were also tested for their ability to grow in IO BG-11 medium and a temperature of 45 ± 1°C, and in other combinations of temperature and salinity. These endolithic organisms have been maintained in the CCMEE culture collection at the University of Oregon in freshwater BG-11 medium at 23 ± 1°C under continuous visible light of 35–65 μE m^{-2} s^{-1} generated by coolwhite fluorescent lamps and also conserved at −80°C. These cultures were originally isolated by Dr. Tracy B. Norris from non-thermal recent and ancient travertine, originally deposited by now extinct calcareous springs in Yellowstone National Park.

Two additional strains of *Leptolyngbya*-like cyanobacteria that showed close similarity (99%) to 16S rDNA sequence alignments of *Iceland Clone 2e* (GR-6 and GR-7) were isolated by R.W. Castenholz from hot springs samples collected at 40–47°C, pH 7.0–7.5, by M. Kühl in 2004 from the eastern Greenland Kap Tobin hot springs (13–16 ppt) on the eastern coast of Greenland (Roeselers et al. 2007).

All cultures used in this study are maintained in the CCMEE that is housed in the Center for Ecology and Evolutionary Biology, University of Oregon (http://cultures.uoregon.edu).

DNA extraction

Genomic DNA extraction from culture isolate (*Iceland Clone 2e*) was performed using a slight modification of the standard method of More et al. (1994) (see procedure in Roeselers et al. 2007). Culture aliquots were pelleted and approximately 100 μL of pelleted cells were transferred to 2 mL screw-cap micro centrifuge tubes. Next, 0.75 g of 0.1 mm diameter zirconium/silica beads (Biospec Products, Bartlesville, OK, USA) were added to the tubes along with 400 μL filter sterilized 120 mM Na-phosphate buffer (pH 8.0) and 200 μL of lysis buffer [10% (wt/vol) sodium dodecyl sulfate, 0.5 M Tris–HCl (pH 8.0) and 0.1 M NaCl]. Cells were lysed by shaking for 4 min at high speed on a mini Bead beater (Biospec Products, Bartlesville, OK, USA) and then centrifuged for 3 min at 13,000×*g*. Supernatant (700 μL) was collected and the DNA precipitated on ice using 2:5 (v/v) of 7.5 M ammonium acetate and then centrifuged again. The supernatant was collected and the DNA was precipitated by isopropanol. Finally the pellets were washed with ice-cold 70% (v/v) ethanol (−20°C); air dried and resuspended in 100 μL of 10 mM Tris (pH 8.0). The extracted DNA was quantified using a Nanodrop spectrophotometer at 260 and 280 nm.

Polymerase chain reaction amplification of rRNA gene fragments of *Iceland Clone 2e*

To amplify the 16S rRNA encoding gene fragments of the cyanobacterium, cyanobacteria-specific primers CYA 359F and CYA 781R (Nübel et al. 1997) were used resulting in a product of 345 bp. Each PCR reaction contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM of each primer, approximately 10 ng of template DNA, 0.1 mg mL^{-1} bovine serum albumin, 2.5 U *Taq* polymerase and 1× PCR buffer (Promega, Madison, WI, USA) in a total volume of 50 μL. The PCR amplification cycle was as follows: initial denaturation for 5 min at 95°C, then 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C, a 1 min extension at 72°C, followed by a final extension of 7 min at 72°C.

Comparative sequence analysis

A portion of the 16S rRNA gene from *Iceland Clone 2e* was sequenced directly in both directions using the original PCR primers with a Beckman Coulter CEQ capillary sequencer at the sequencing facility (Eugene, OR, USA). The sequence data were assembled in BioEdit Sequence Alignment Editor v, 5.0.9 (Hall 1999), and nucleotide alignments of 16S rRNA gene sequence for the novel sequence and several other cyanobacterial taxa were created using CLUSTALX with default gap penalties (Higgins and Sharp 1988, 1989; Thompson et al. 1997). Gaps were excluded for phylogenetic analyses. Distance matrix trees were generated by the neighbor joining method as implemented in PAUP* 4.0B (Sinauer, Sunderland, MA, USA). The NJ calculations were subjected to bootstrap analysis (1,000 replicates). Maximum parsimony analysis was performed in PAUP using the heuristic search option with 100 repetitions of random sequence addition and was bootstrapped 100 times. The maximum likelihood analysis was performed using PHYML on-line (Guindon and Gascue 2003; Guindon et al. 2005), and bootstrapped 100 times. Model parameters for the neighbor joining and maximum likelihood analysis were set to conform to the general time reversible model with gamma distribution and proportion of variable sites as indicated by Modeltest version 3.06 (Posada and Crandall 1998). *Chloroflexus aurantiacus* DSM 637 and *Escherichia coli* APEC O1 were used as the outgroups for all analyses.

Growth experiments and pigment extraction

Cultures of 5 of the 6 *Leptolyngbya*-like strains were grown in 80 mL of medium in 125 mL Erlenmeyer flasks and maintained at 45 ± 1°C (23–24°C for CCMEE 6111), swirled several times per day, and illuminated continuously

from below (coolwhite fluorescence at ~150 μE $m^{-2}s^{-1}$, measured by Biospherical Instruments QSL-100, San Diego, CA, USA). Exponentially growing cultures acclimated at this temperature for 15 days were used to inoculate triplicate experimental flasks. The trichomes of all 6 *Leptolyngbya* strains had only a slight tendency to clump together or attach to vessel walls. Very thin diaphanous sheaths surrounded some of the trichomes. Standard microbiological culture and experimental techniques of cultures that grow in suspension could not be used in this case. Under sterile conditions the cultures in each flask were well homogenized with the help of a sterile 10 mL syringe with an elongate sterile cannula, after any material sticking to the glass was suspended by wiping with a sterile, latex "policeman" at the tip of a glass rod. Experiments were usually carried out for 120 h. Subsamples of 5 mL were withdrawn every 2 h for a total of 15 time points during the apparent exponential phase of strains with rapid growth, and at longer intervals during slow growth. Several earlier trials determined the optimal time intervals. The samples were filtered and washed on 25 mm Whatman GF/C glass fiber filters. While still moist, chlorophyll and carotenoids were extracted by placing the filter at the bottom of a 20 mL scintillation vial loaded with 5 mL of absolute methanol saturated with $MgCO_3$. The scintillation vials were kept at 4°C overnight in the dark. The glass vials were swirled a few times near the beginning and end of extraction. Care was taken to not disturb the settled particulates at the bottom of the vial before removing the clear extract and reading the optical density. The absorbance of the clear extract was measured at 665 nm (corresponding to the chlorophyll *a* peak) against a similarly clarified methanol blank with $MgCO_3$, using a Beckman DU 640 recording spectrophotometer. Growth curve slopes were determined by log-linear regression of changes in chlorophyll content over time during the apparent exponential growth period and doublings per day (k) were estimated as follows: $k = \log_2 (N_t/N_0)/\Delta t$, where N_t is the Chl *a* absorbance at the end of the exponential phase in days (t) and N_0 is the Chl *a* absorbance at the beginning of exponential phase in days. In all experiments, the 3 culture flasks received identical inoculum at the beginning of each experiment.

Effect of salinity on growth

The saltwater medium (IO BG-11) containing approximately 28 g L^{-1} TDS (as BG-11 plus "Instant Ocean™") was used for the initial growth experiments. Medium with increased salinities of 66 and 94 g L^{-1} was attained by adding additional NaCl. The last 2 salinities correspond to approximately twice and 3 times the salinity of normal seawater. A range of salinities from 28, 66, 94, and 132 g L^{-1} TDS was initially tested for survival and growth of the 6 strains of *Leptolyngbya*, but none survived at 132 g L^{-1} at any temperature from 23 to 54°C. Growth rate experiments used the same procedure as above. In all experiments triplicate cultures were used, and the results of the repeat trial are shown.

Effect of temperature on growth

Survival and growth of the *Leptolyngbya*-like strains were examined at 23, 45 ± 1, 50, 54, and 58 ± 1°C, to ascertain what temperatures were optimal, suitable, or lethal. Experiments were conducted at each temperature in incubators set to the particular temperature but under the same light intensity of ~150 μE m^{-2} s^{-1}. In all cases, at the different temperatures growth was tested at 66 and 94 g L^{-1}. Growth was followed as per procedures described above and doublings per day calculated in each case.

Microscopy

Collection and cultures were examined using an American Optical research microscope with phase contrast 40× and 100× objectives. Photomicrographs were made with a Zeiss Axioplan microscope, using a 100× plan neofluor objective with Nomarski DIC. Photo-images were made with a Nikon Coolpix digital camera. The taxonomic descriptions and tentative generic names used in *Bergey's Manual of Systematic Bacteriology* (Castenholz et al. 2001) were used for morphotypic identifications.

Statistical analysis

A 4 parameter logistic curve was fit for absorbance as a function of time for each combination of temperature and salinity using the drc package (Ritz and Streibig 2005). See Results (Growth: effects of salinity and temperature for *Iceland Clone 2e*) for further details.

Results

Characteristics of the thermal, saline field site

The Blue Lagoon (Bláa Lónid) collection site is located at 63°52′48.31″N, 22°26′55.19″W on the Reykjanes Peninsula, southwestern Iceland. The saline warm waters of the Blue Lagoon arise from altered thermal brines that are associated with a terrestrial exposure of the Mid-Atlantic Ridge separation of the European and North American tectonic plates (Tómasson and Kristmannsdóttir 1972; Gudmundsson et al. 1981, Pétursdóttir and Kristjánsson 1996). The geothermal brines are very deficient in Mg and sulfate, but are

significantly enriched in K and Ca, relative to seawater. However, the silicate content of the thermal brines at about 70°C was often almost 100× that of seawater (Gudmundsson et al. 1981) and polymerized silica precipitated as a crust and soft sediment when the water cooled to 40–45°C (Fig. 1). Thus, this habitat is unique. At the time of collection in August, 2006, the salinity of the Blue Lagoon water was measured at ~32 g L^{-1} TDS and the pH at ~5.7–6.3. The Greenland geothermal field sites are typical of terrestrial hot springs (Roeselers et al. 2007), with the isolates of interest from the Kap Tobin springs with a TDS content of 13–16 g L^{-1}. The non-thermal collection sites of extinct travertine spring deposits in Yellowstone National Park are described in Norris and Castenholz (2006).

Phylogenetic comparisons of the study strain in relation to other cyanobacteria

Neighbor joining, maximum likelihood and maximum parsimony phylogenetic analyses of the cyanobacteria were prepared using partial 16S rRNA gene sequences, mainly using cyanobacterial-specific primers (see "Materials and methods"). In all analyses, the study strain *Leptolyngbya* (*Iceland Clone 2e*) grouped with strong support with the *Leptolyngbya*-like isolates from the Kap Tobin springs (Greenland), the 2 environmental sequences from the Greenland spring, and the 3 endolithic cultures from YNP (NJ tree shown in Fig. 2). Only in the case of the 2 Greenland cultures were the complete 16S rRNA gene sequences of 1,485 bp available (Roeselers et al. 2007). The moderately thermophilic–halophilic *Halomicronema* (Abed et al. 2002) did not affiliate with this clade in any analysis (Fig. 2). The *Iceland Clone 2e* (*Leptolyngbya*) carries accession number EF539879 in the NCBI database.

Growth: effects of salinity and temperature

Iceland Clone 2e

A single test experiment was run to verify that chlorophyll *a* absorbance increased during exponential phase in parallel to dry weight. When *Iceland 2e* culture was grown at 45°C in IO BG-11 medium the exponential rate, using chlorophyll *a* absorbance, was 3.3 doublings 24 h^{-1}. In the parallel experiment beginning with the same inoculum, using increases in dry weight (measured on pre-weighed desiccated Nuclepore filters with a Mettler M-5 micro-balance) the doubling rate was calculated at a very similar 3.2 doublings 24 h^{-1} (data not shown).

An axenic, clonal culture of *Leptolyngbya* (*Iceland Clone 2e*, from the Blue Lagoon) was evaluated with respect to survival and growth in different media [BG-11, IO BG-11 (~28 g L^{-1} TDS), and IO BG-11with various additions of NaCl], and at different temperatures. *Iceland Clone 2e* was 100% identical at the 16S level to an earlier strain isolated from the Blue Lagoon by J. Kristjánsson (personal communication). That strain was tentatively identified as *Leptolyngbya erebi* var. *thermalis* by Dr. A. Couté, on the basis of morphology (Pétursdóttir and Kristjánsson 1996).

A general linear model was used to determine the overall influences of temperature and salinity on growth rates of *Iceland Clone 2e*. Both temperature ($F_{2,447} = 172.49$, $p < 0.0001$) and salinity ($F_{2,447} = 160.21$, $p < 0.0001$) had a significant effect on growth rate, as expected (Tables 1, 2, 3). When holding salinity constant there was a general trend for a reduction in growth rate and a reduction in the final yield of chlorophyll *a* as temperature increased (Table 1). When temperature was held constant the significant effect of salinity may be seen, by noting that in 2 of the 3 cases maximum growth rate and yield occurred at 45°C and 66 g L^{-1} TDS (Table 1). The interaction between salinity and temperature may be seen when the temperature was 54°C. In this case the growth rate was maximized when the salinity was 28 g L^{-1} TDS.

This cyanobacterium demonstrated little or no growth in freshwater BG-11 medium at 23, 45, 50 and 54°C; there was no survival at 58°C (Table 2). Also, the growth rate was extremely slow at 23°C (<0.05 doubling 24 h^{-1}) even when grown in IO BG-11 medium (~28 g L^{-1} TDS) (Table 2). Growth rate was greatly enhanced at 45°C in saline IO BG-11 medium (Tables 1, 2). Growth rates showed an exponential doubling rate of 3.8 per 24 h under continuous light (Tables 1, 2). The growth rate at 45°C was slightly enhanced (a 15% increase) at 66 g L^{-1} TDS to 4.4 doublings 24 h^{-1} (Table 1). At 94 g L^{-1} TDS, however, the growth rate was reduced to 3.3 doublings 24 h^{-1} at 45°C and the final yield was lower (Table 1). The final yield paralleled in most cases the growth rate, i.e., it usually decreased as growth rate fell (Table 1).

Iceland Clone 2e was further studied for its ability to grow and survive at higher temperatures (50, 54 and 58°C) at various salinities. The growth rate at 50°C was very similar to that at 45°C at all 3 salinities, but the yield was less at 94 g L^{-1} TDS (Table 1). With a further increase in temperature to 54°C the growth rate decreased measurably when compared to that at 50°C, but at each temperature the growth rate was similar at all 3 salinities but the final yield fell considerably at 94 g L^{-1} at all temperatures (Table 1). The reductions in growth rates at 66 g L^{-1} at 50 and 54°C, when compared to 45°C, were 19 and 56%, respectively, although growth at 50°C in 94 g L^{-1} TDS did not show this decrease (Table 1). The final yield for all 3 salinities was also much reduced at 54°C when compared to 45 or 50°C (Table 1). *Iceland Clone 2e* died at 58°C at all salt concentrations, as realized by complete bleaching.

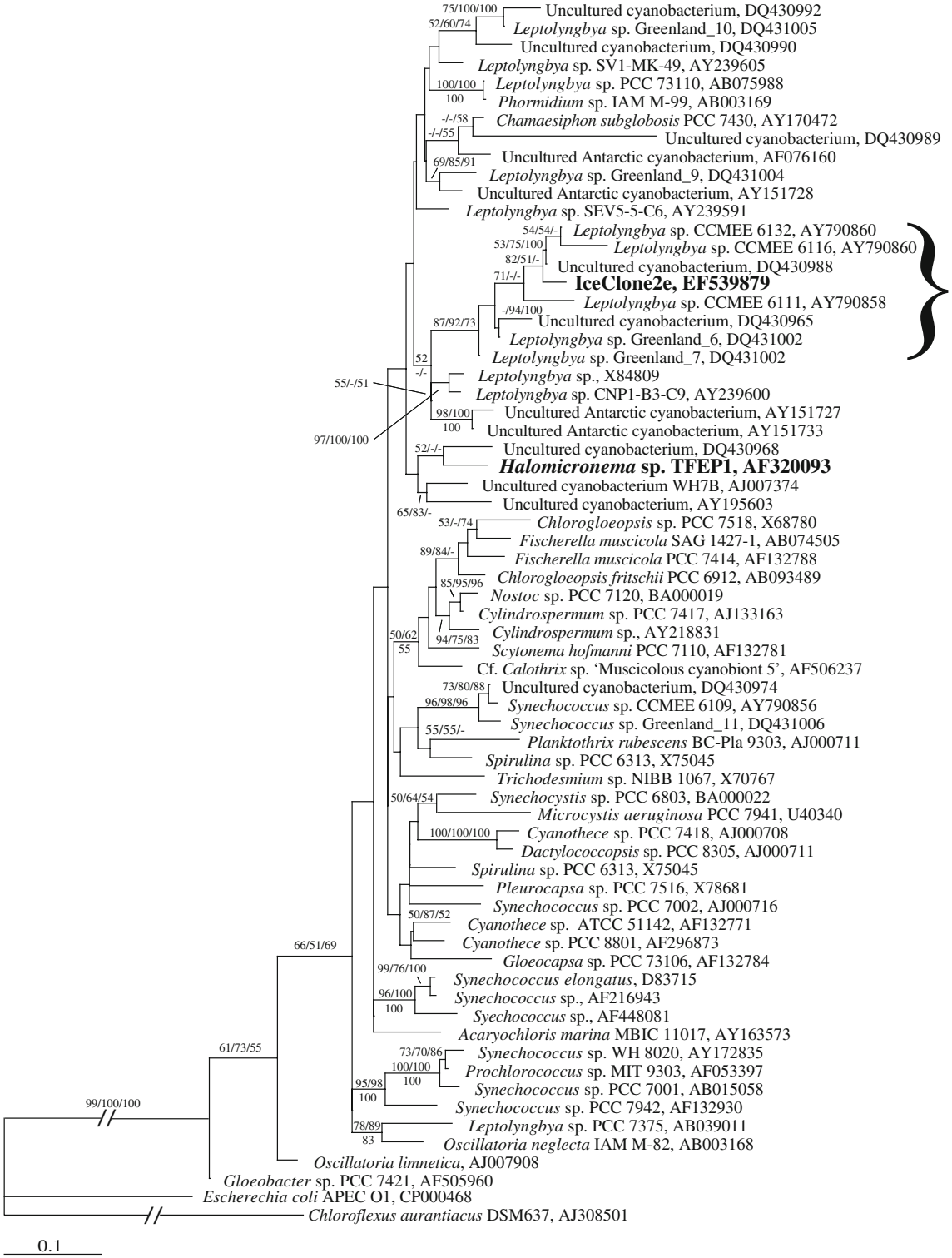

Fig. 2 Neighbor-joining (*NJ*) phylogram of the cyanobacteria inferred from partial 16S rDNA sequence data. Bootstrap values are shown for **NJ/ML/MP**, respectively. *Hyphens* indicate boostrap values of >50. *Escherichia coli* and *Chloroflexus aurantiacus* are used as outgroups. The *bracket* indicates the cluster or clade of strains or environmental sequences that were involved in this study. The newly isolated thermophilic–halophilic *Iceland Clone 2e* (**Ice Clone 2e**) is in *bold*. All of those cultures designated by CCMEE are from travertine rocks in YNP and are located in the University of Oregon Culture Collection of Microorganisms from Extreme Environments (Norris and Castenholz 2006) The DQ preface within the *bracketed clade* indicates cultures or sequences from the east Greenland hot spring system. All the Greenland hot spring cultures [**Greenland_6 (GR-6)** and **Greenland_7 (GR-7)**] also reside in the CCMEE at the University of Oregon (Roeselers et al. 2007). The other described thermo-halophilic cyanobacterium, *Halomicronema*, is in *bold* (Abed et al. 2002)

Table 1 Doublings 24 h^{-1} of *Leptolyngbya Ice Clone 2e* in different salinities as g L^{-1} TDS at 45, 50 and 54°C

°C	28 g L^{-1}	Final yield	66 g L^{-1}	Final yield	94 g L^{-1}	Final yield
45	3.8	0.387 ± 0.008	4.4	0.436 ± 0.005	3.3	0.161 ± 0.008
50	3.5	0.336 ± 0.007	3.5	0.441 ± 0.007	3.6	0.152 ± 0.008
54	2.2	0.203 ± 0.006	1.9	0.139 ± 0.005	2.0	0.112 ± 0.006

The final yield is presented as chlorophyll *a* absorbance at 665 nm, with SE indicated

Table 2 Growth responses of *Ice Clone 2e* and related *Leptolyngbya* strains in freshwater BG-11 and saline IOBG 11 medium at different temperatures (doublings 24 h^{-1})

Leptolyngbya strain	23°C		45°C		50°C		54°C	
	BG 1I	IOBG11	BG11	IOBG11	BG11	IOBG11	BG11	IOBG11
Ice. Clone 2e	0	+	+	3.8	+	3.5	+	2.2
6132	0.5–1.0	+	+	0.7	Dns	0.7	Dns	Dns
6116	0.5–1.0	+	+	2.2	Dns	2.1	Dns	Dns
6111	0.5–1.0	1.0	Dns	Dns	Dns	Dns	Dns	Dns
GR-6	+	+	+	3.3	+	2.4	Dns	Dns
GR-7	+	+	+	2.4	+	2.4	Dns	Dns

Dns did not survive

+ Slow growth, <0.05 doublings 24 h^{-1}

0 No growth, survival dubious

Table 3 Comparative growth rates of *Leptolyngbya* clones 6132, 6116, GR-6 and GR-7 in different salinities at 45 and 50°C shown as doublings 24 h^{-1}

Temperature °C	28 g L^{-1} TDS	66 g L^{-1} TDS	94 g L^{-1} TDS
(a) *Leptolyngbya* strain 6132 (Yellowstone travertine)			
45	0.7	0.7	0.6
50	0.7	Dns	Dns
(b) *Leptolyngbya* strain 6116 (Yellowstone travertine)			
45	2.2	1.5	Dns
50	2.1	Dns	Dns
(c) *Leptolyngbya* strain GR-6 (Kap Tobin Hot Spring, Greenland)			
45	3.3	1.9	Dns
50	2.4	1.6	Dns
(d) *Leptolyngbya* strain GR-7 (Kap Tobin Hot Spring, Greenland)			
45	2.4	Dns	Dns
50	2.4	Dns	Dns

None survived at 54°C or higher

Dns did not survive

Greenland hot spring cultures, GR-6 and GR-7 (Greenland_6 and Greenland_7)

The 2 Greenland isolates, GR-6 and GR-7, that were related to *Iceland Clone 2e* (Fig. 2) did not show growth at 23°C in either BG-11 or IO BG-11 medium (Table 2). Neither strain tolerated 54°C or a higher temperatures at any salinity. They differed from each other, however, in their responses to salinity at 45°C. GR-6 showed a doubling 24 h^{-1} of 3.3 in saline IO BG-11 medium (Table 3c), and this rate declined with the increase in salinity to 66 g L^{-1} TDS, a reduction of ~41% (Table 3c). GR-7 grew only in IO BG-11 at 45 and 50°C with a doubling rate of ~2.4 doublings 24 h^{-1} (Tables 2, 3d). However, it was unable to tolerate 66 g L^{-1} at any temperature (Table 3d). Neither GR-6 nor GR-7 tolerated 94 g L^{-1} TDS at any temperature.

Endolithic cyanobacteria from Yellowstone National Park

Leptolyngbya sp. CCMEE 6132 was obtained originally from terrestrial (subaerial) travertine (Norris and Castenholz 2006). It grew well at 23°C in freshwater BG-11 medium, as expected, but poorly at that temperature in IO BG-11 (Table 2). However, it showed a doubling time of 0.7 per 24 h in IO BG-11 at 45 and 50°C (Tables 2, 3). It also grew at 66 and 94 g L^{-1} TDS at 45°C (Table 3a). Remarkably, it grew at 50°C in saline medium (IO BG-11) but not at 50°C in freshwater BG-11 medium (Table 2).

Leptolyngbya sp. CCMEE 6116 (also isolated from ancient travertine) showed much higher growth rates at 45 and 50°C in IO BG-11 medium, i.e., about 2 doublings 24 h^{-1} at both temperatures (Tables 2, 3b). It also grew well in 66 g L^{-1} TDS at 45°C (1.5 doublings 24 h^{-1}) (Table 3b). As in the case of CCMEE 6132, growth at 50°C took place only in saline medium (Table 2).

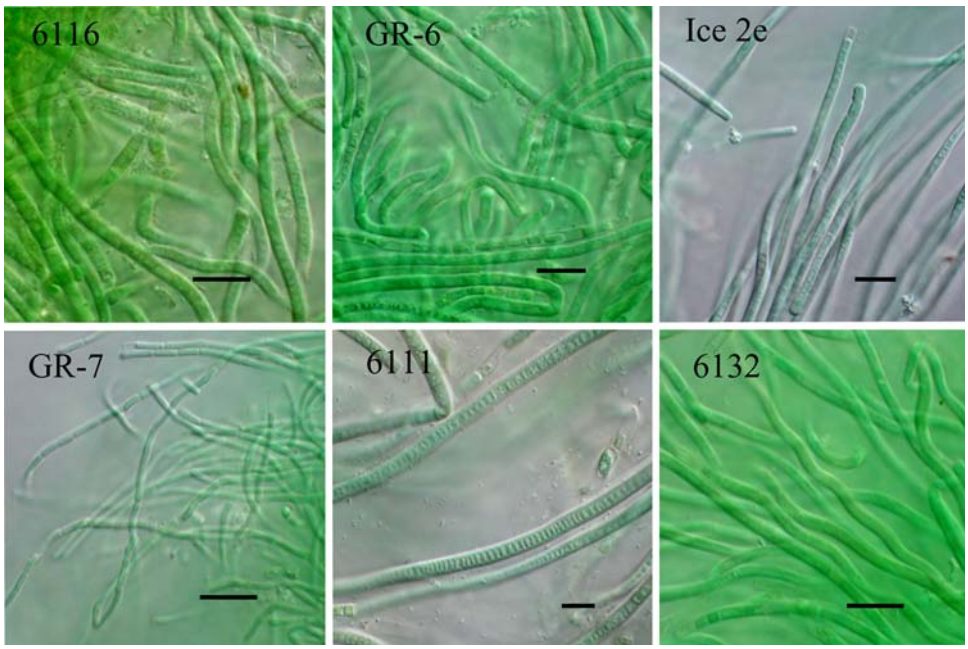

Fig. 3 Photomicrographs of the 6 culture strains used in this study at approximately the same stage of growth (late exponential-early stationary) at 45°C and in IOBG-11 medium (except for CCMEE 6111 grown at 23°C, since growth did not occur at 45°C). The *bars* are approximately 5 μm

Leptolyngbya sp. CCMEE 6111 (also from ancient travertine and usually grown at 23°C in BG-11 medium) also grew on IO BG-11 medium at 23°C with a growth rate of ~1.0 doublings 24 h^{-1} (Table 2). This strain, however, was unable to survive at 45°C in all salinities.

It was also of interest and relevant to test other *Leptolyngbya*-like endolithic strains from Yellowstone, even though they were not phylogenetically included in the clade with *Iceland Clone 2e* (see Norris and Castenholz 2006). These also were normally grown at 23°C in freshwater BG-11 medium but also survived and grew in IO BG-11 medium at 23°C. However, only 5 out of 20 strains survived and grew (but slowly) in this medium at 45°C. Three of these also grew slowly at 50°C in the saline medium. In freshwater medium only one strain survived at 45°C, but not at 50°C (data not shown).

Desiccation and freezing tolerance of the 6 *Leptolyngbya* strains

It was also of interest to see if other phenotypic characteristics of these *Leptolyngbya*-like isolates were shared. Two of the strains related to *Iceland Clone 2e* that were endolithic cyanobacteria from travertine rock in Yellowstone Park (CCMEE 6132 and 6116) were able to survive 7 days of desiccation at 19% relative humidity and 7 days of freezing at −15°C, in both cases with recovery at 45°C without subsequent bleaching. CCMEE 6111 also survived freezing, but after desiccation and rewetting showed fractional survival, i.e., some bleaching occurred (data not shown). *Iceland Clone 2e* was able to survive desiccation and freezing when grown in IO-BG11 medium and recovered in the same medium at 45°C, without any bleaching and with outgrowth in fresh medium within 3–4 h. GR-6 and GR-7 also survived desiccation and freezing, with rapid recovery and no bleaching in BG-11 and IO-BG11 medium at 45°C (data not shown).

Microscopic morphology of the 6 *Leptolyngbya* strains

Trichome diameter is a fixed character in most of the order Oscillatoriales, whereas cell length is quite variable and is mostly dependant on rapidity of cell division and growth, although some enlargement in trichome diameter occurred in these strains under stress or in late stationary growth phase. *Iceland Clone 2e* trichomes, during active growth, showed a diameter of 2.1–2.2 μm (Fig. 3). CCMEE 6132, CCMEE 6116, and GR-6 were about the same diameter or slightly wider (Fig. 3). CCMEE 6111 (the strain least similar with respect to temperature and salinity tolerances and optima) was considerably wider (3.0–3.3 μm) with very short cells (Fig. 3). GR-7, however, from the same Greenland hot spring as GR-6, had a trichome diameter range of 0.8–1.0 μm (Fig. 3), only about half the diameter of GR-6 and *Iceland Clone 2e*. Cells, except for those in strain 6111, were longer than broad, but, as usual, the lengthwise dimension varied with the age of the culture. Barely perceptible, thin diaphanous sheaths were occasionally seen in all strains. It is apparent that 16S rRNA gene sequence similarities do not invariably determine morphological similarities.

Discussion

The ability of the *Leptolyngbya*-like isolate from the warm saline waters of the Blue Lagoon in Iceland to grow well in warm (45–54°C) waters at salinities at and well above seawater salt concentrations was a novel finding at least as far as physiological temperature/salinity data are concerned. A thermo-halophilic cyanobacterium has apparently been described only once before in any detail (Abed et al. 2002), and that species, *Halomicronema excentricum*, appears rather distantly related to the cyanobacteria of the present study (Fig. 2). Moderately thermophilic and highly halotolerant, unicellular *Aphanothece* strains were also described, but without genetic data (Dor and Hornoff 1985).

A major revelation of the present study is that the *Iceland Clone 2e* formed part of a well-supported phylogenetic clade that included other *Leptolyngbya*-like cultures that were isolated from normally dry endolithic sites in travertine rocks in the Mammoth region of Yellowstone National Park in the western United States (Norris and Castenholz 2006) (Fig. 2). The present clade (based on 16S rDNA sequence comparisons) also included 2 related Greenland isolates from low salinity terrestrial hot springs in east Greenland as well as 2 environmental 16S rDNA sequences obtained by DGGE from the same Greenland hot springs. These springs are the only thermal springs in Greenland with source waters above 45°C (Roeselers et al. 2007).

Another unexpected finding was that most of the related culture strains from different habitats, also possessed some comparable key phenotypic properties related to temperature and salinity ranges, tolerances, and morphology. Several other cyanobacterial strains have shown close similarities, using 16S rDNA sequence comparisons, but have not been very similar in several physiological or morphological traits (e.g., Rocap et al. 2003, Logares et al. 2007). Of course, there may be numerous other phenotypic and genotypic differences among the strains of the present study, but because of the focus in this report, mainly thermo-halophilic characteristics were described.

An astounding finding of this study was that 2 of these cyanobacteria from Yellowstone travertine grew as thermophiles only in saline medium, although they were obtained from non-thermal, non-saline habitats. Salinity apparently is a major factor stimulating growth at 45°C (or higher temperature) of 5 strains of this study. The basis for this phenomenon is unknown. It is apparent from the data we generated that optimal growth conditions for Greenland and Yellowstone strains (except CCMEE 6111), to be 45–50°C in IO BG-11 medium ($\sim$28 g L^{-1} TDS) (Tables 2, 3) and, in the case of *Iceland Clone 2e*, the optimum extended to even higher salt concentrations, i.e., to 94 g L^{-1} TDS at 45 and 50°C (Table 1). A somewhat similar finding was that in the unicellular halotolerant cyanobacterium, *Aphanothece halophytica* growth was stimulated at the highest temperature of 48°C by increased salinity (Dor and Hornoff 1985). Garcia-Pichel et al. (1998) also found with unicellular halophiles that increased salinity benefited growth at higher temperatures (38°C) more than at 25°C.

The rate of 3–4 doublings 24 h^{-1} characterizes the *Iceland Clone 2e* as a very fast growing cyanobacterium. Few filamentous forms of cyanobacteria are known to grow this rapidly, especially under the moderate light intensity of 150 μE m^{-1} s^{-1}. Some unicellular thermophilic forms (e.g., *Synechococcus*) from terrestrial hot springs show a similar doubling rate under a comparable light intensity (Miller and Castenholz 2000).

Chlorophyll *a* increase as a proxy for growth is commonly used, and, at least during exponential growth phase, usually corresponds to increase in cell mass. This was shown here in 1 parallel experiment in which cell dry weight was used, although chlorophyll itself is an important standard. In general, a lower final cell yield in the case of bacteria correlates well with lower growth rates, but the basis of this is often not understood (Table 1).

The phylogenetic similarity at the 16S rRNA gene locus of all the strains studied here indicate that they have probably evolved from a common ancestor and spread and diverged into discrete species or strains in varied and distinctly dissimilar habitats over a great distance geographically over what was presumably a very long geologic time scale. There are about 8,000 direct km between Iceland and Yellowstone geothermal springs. However, there is no reason to assume that the distribution data for this group of *Leptolyngbya*-like cyanobacteria are complete, and that all potential *Leptolyngbya*-like strains or species in this clade are in a database.

The fact that there are some similarities, with respect to salinity and temperature tolerances, between strains from endolithic habitats and *Iceland Clone 2e* suggests that the hot saline habitat of the Blue Lagoon in Iceland bears some similarity to conditions that occur periodically in the endolithic habitat of rocks, such as the travertine of Yellowstone. The endolithic cyanobacteria studied from the YNP travertine form a thin 1–2-mm thick greenish band 1–2 mm below the rock surface (Norris and Castenholz 2006). As drying conditions develop during summer, or after a summer rain, these cyanobacteria, in a few cases examined, would have experienced both high temperature ($\sim$45°C) and possibly increasing salinity through evaporation. Thus, the physiological conditions encountered by these endolithic forms may at times be somewhat similar to those realized by cyanobacteria of the Blue Lagoon. Unfortunately, critical measurements within the travertine

during these periods of heat and developing desiccation stress have not been made.

Acknowledgments M. B. gratefully acknowledges the Department of Biotechnology, Ministry of Science and Technology, Government of India for the DBT Overseas Associateship that permitted this visit from Barkatullah University, Bhopal, India to the Department of Biology, Center for Ecology and Evolutionary Biology, University of Oregon, USA. Statistical analyses were provided by Kevin Emerson and we are grateful for his contribution. We also thank Dr. Jakob Kristjánsson (University of Iceland) for facilitating the collection of microorganisms from hot springs in Iceland and for discussions relating to the Blue Lagoon.

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