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Ecophysiology of a Mono Lake picocyanobacterium

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Abstract

We isolated an unusual picocyanobacterium (strain MLCB) from alkaline, hypersaline Mono Lake, California. The organism blooms ($\sim5.0 \times 10^7$ cells L^{-1}) in late summer. It has very low population densities in the photic zone through spring and summer ($<10^5$ cells L^{-1}) but maintains a significant population (10^7–10^8 cells L^{-1}) in anoxic, sulfidic waters below 25 m year-round during meromictic periods. Complete turnover of the lake resulted in a significant (>90%) loss of the deep-water population that could not be attributed to simple dilution. The deep-water population returned to its previous concentration by the following autumn. The organism is phycoerythrin rich, does not contain phycourobilin, and is not capable of complementary chromatic adaptation. Phylogenetically, our isolate groups with marine *Synechococcus*, *Prochlorococcus*, and *Cyanobium* genera. Salinity tolerance of our strain was compared to select members of the *Cyanobium* and showed that strain MLCB was the most halotolerant, capable of growing at 10% salinity, compared to limits of 0–6% for the other strains tested.

Mono Lake lies on the eastern edge of the Sierra Nevada mountain range, at the western border of the Great Basin. It is fed primarily by melting snowfall via a series of alpine lakes and streams. The lake is endorheic, and the basin's volcanic setting (Lajoie 1968) includes numerous hydrothermal springs that contribute to the lake's unique water chemistry (Table 1). Mono Lake is subject to recurrent periods of meromixis (persistent chemical stratification; Fig. 1) as a result of natural and anthropogenic alterations of freshwater flow into the lake (Jellison et al. 1998), with the most recent episodes persisting from 1995 until 2003 and from 2005 until the present.

Eukaryotic phototrophs, mainly a strain of the green alga *Picocytis salinarum* and diatoms, are responsible for the majority of primary production in Mono Lake (Jellison and Melack 1988). Phytoplankton productivity is high (350 to >1,000 g C m^{-2} yr^{-1}) with marked seasonal cycles of abundance (Jellison and Melack 1988, 1993*a*; Roesler et al. 2002). Eukaryotic algae are abundant throughout the winter, and their biomass increases substantially in spring as the thermocline stabilizes. Grazing by the brine shrimp *Artemia monica* during summer rapidly reduces algal standing crops in the upper water column (Fig. 1C) and results in a pulse of sinking, labile organic matter. This carbon fuels respiration in bottom water and results in oxygen depletion and anoxia during late spring and summer (Fig. 1B). During prolonged stratification, bottom waters accumulate high concentrations of reduced inorganic compounds such as sulfide (Fig. 1C), ammonia, and arsenite (Hollibaugh et al. 2005). In contrast to many other soda lakes, widespread anoxygenic photosynthesis does not result in the formation of a subsurface maximum of microbial biomass (a "plate") in Mono Lake, likely because of the depth of the mixed layer and low irradiance (Fig. 1D) at depths containing sulfide (Cloern et al. 1983). Instead, the "plate" that develops at the bottom of the thermocline in summer is dominated by *Picocystis* (Hollibaugh et al. 2001; Roesler et al. 2002). Given this distribution of phototrophs, we were surprised to find relatively high populations of unicellular cyanobacteria in the waters below the "plate," especially since they were not detected in surface waters of the lake.

Picocyanobacteria (Pcy) are ubiquitous in aquatic environments and are important contributors to global carbon fixation. Their small size (0.2–2 μm) results in increased surface-to-volume ratios, allowing them to reduce the limitations of nutrient acquisition set by molecular diffusion and gain an advantage over larger phytoplankton in low-nutrient environments (Raven 1998). However, they also compete well in eutrophic environments, with some studies showing Pcy contributing 30–90% of the total production (Maeda et al. 1992; Carrick and Schelske 1997).

Phylogenetically, Pcy of the marine *Synechococcus*, *Prochlorococcus*, and *Cyanobium* genera form a tight clade within the cyanobacteria (Urbach et al. 1998; Wilmotte and Herdman 2001). Currently, there are >50 phylogenetically distinct isolates within the genus *Cyanobium*, representing a high species diversity (Crosbie et al. 2003; Ernst et al. 2003). In contrast to marine *Synechococcus* and *Prochlorococcus*, *Cyanobium* (and other Pcy) ecotypes are found in many distinct and contrasting habitats, and they have not been shown to contain the pigments phycourobilin or chlorophyll *b*. This suggests that the environmental factors influencing the speciation of the individual Pcy groups will most likely differ. The factors shaping niche development in marine Pcy are well documented, such as the form of N available to them (Moore et al. 2002), light intensity and wavelength (Palenik 2001), and even motility (Toledo et al. 1999). In general, less is known about the genetic and physiological diversity of Pcy from inland and coastal

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Table 1. Characteristics of Mono Lake.

Area (km^2)	180
Volume (km^3)	3.3
Max/mean depth (m)	47/18
Salinity	8.5%
pH	9.8
Nitrogen (NH_4) (μmol L^{-1})	0–15* to >500†
Phosphorus (SRP) (μmol L^{-1})	500
DOC‡ (mg L^{-1})	50*–300†
Carbonate (mmol L^{-1})	400
Sulfate (mmol L^{-1})	130
Sulfide (μmol L^{-1})	0* to >3,000†
Arsenic (μmol L^{-1})	200

* Epilimnion.
† Beneath chemocline.
‡ Dissolved organic carbon.

waters. Similar to marine Pcy, the light environment has been shown to influence the pigment content of two similar *Cyanobium* isolates from the Bornholm Sea (Ernst et al. 2003). Other factors, such as salinity, sulfide concentration, eutrophication, and trace metal concentrations, could contribute to speciation in freshwater Pcy ecotypes.

Cyanobacteria are barely detectable in the photic zone of Mono Lake for most of the year and contribute little to primary production. However, we found a single Pcy ecotype that was abundant (10^7 cells L^{-1}) in the anoxic, sulfide-rich bottom waters of the lake. *Cyanobium* species are considered halotolerant, but they have not been reported at salinities above 5%. Nor is their presence in aphotic lake waters uncommon, especially in meromictic lakes, but the means by which these populations are maintained is subject to speculation (Craig 1987; Detmer et

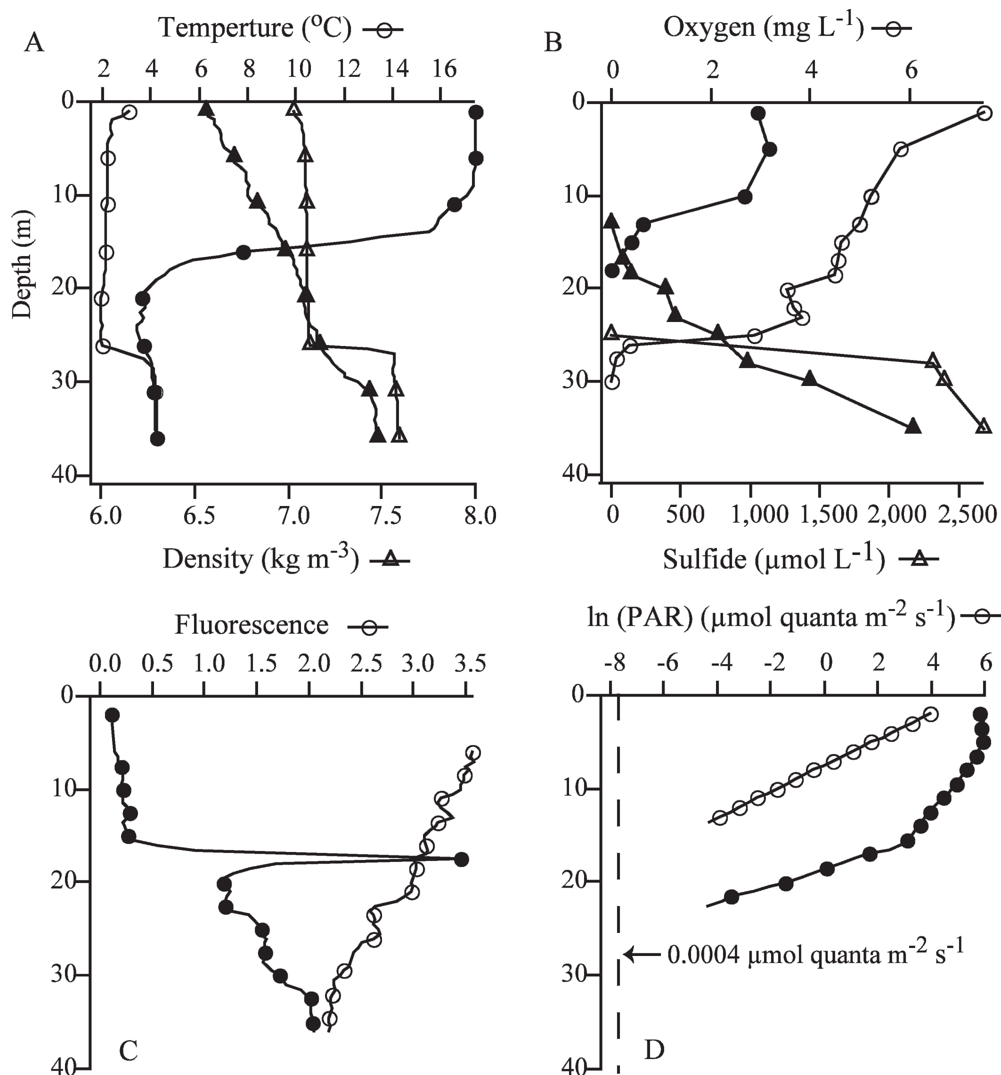

Fig. 1. Vertical profiles contrasting summer (closed symbols) and winter (open symbols) conditions in Mono Lake. (A) Temperature and density. (B) Oxygen and sulfide. (C) Fluorescence. (D) Photosynthetically active radiation. Panels A, C, and D are from high-resolution CTD casts; every tenth datum is shown as a symbol. The vertical dashed line on panel D indicates the lowest light level where growth by phototrophic bacteria has been suggested (Manske et al. 2005).

al. 1993; Malinsky-Rushansky et al. 1997). The puzzle posed by the presence of an apparently healthy population of Pcy in the aphotic, anoxic, sulfidic, and hypersaline bottom waters of Mono Lake motivated this study. This distribution raises interesting ecophysiological questions, particularly how the population is maintained without light and how it tolerates anoxia and elevated salinity. Here we present data on the organism's seasonal distributions and examine the physiology of an isolate to elucidate its autecology.

Methods

Sample collection—Water samples used in this study were collected at various depths (2–40 m) from the central basin of Mono Lake at Station 6 (37°57.822′N, 119°01.305′W). Bathymetric charts of the lake are presented in a number of publications (cf. Jellison and Melack 1993*b* and http://geopubs.wr.usgs.gov/map-mf/mf2393/). Bathymetric data were used to construct a hypsographic curve for the lake (Pelagos 1987). Sampling occurred over a period of 4 years (September 2001–September 2005); however, the majority of the data presented here are from 2001 and 2002 because monthly sampling during this period gave a more thorough description of seasonal changes in Pcy abundance. Additional sampling in 2003 and 2004 revealed the same overall patterns of abundance. Vertical profiles (Fig. 1) of temperature, pressure, conductivity, photosynthetically active radiation (400–700 nm), and fluorescence (chlorophyll: excitation 460 nm; emission 695 nm) were obtained with a SeaBird SBE 19 SeaCat profiler equipped with a Licor 2π sensor and a WetLabs WetStar fluorometer. Oxygen profiles were obtained with a polarographic oxygen sensor (YSI) equipped with a Clark-type electrode. Sulfide was measured using an electrode that measures total free sulfide (Thermo-Orion Method #94-16). Samples (4.5 mL) were taken using a gastight syringe and injected into 5.0 mL of sulfide antioxidant buffer (Orion 941609) as described previously (Hollibaugh et al. 2005).

Pcy enumeration—Samples for Pcy enumeration were collected at depth with a Niskin bottle, placed in airtight plastic bottles, and kept in the dark at 4°C until they were counted (most within 2–3 days, all within 10 days; time course studies indicated no change in abundance over this period of time). Cyanobacteria were enumerated using a Leica epifluorescence microscope containing filter sets N2.1 (excitation 515–560 nm), A (excitation 340–380), and I3 (excitation 450–490), allowing for the differentiation of cells containing phycoerythrin from those containing only phycocyanin.

Isolation of strain MLCB—Samples of anoxic Mono Lake bottom water containing the highest concentration of Pcy (10^8 cells L^{-1}) were chosen for enrichment. Placing Mono Lake bottom water in the light encourages the growth of *Picocystis*, making it extremely difficult to isolate other phototrophs. Consequently, during phototrophic isolation of the Pcy, the culture medium was amended with cycloheximide (5–10 mmol L^{-1}) to inhibit growth of eukaryotic algae. We succeeded in isolating a Pcy ecotype, subsequently designated strain MLCB, into unialgal culture (using "unialgal" here in the broadest sense to include cyanobacteria) by this procedure. In general, the organism did not grow well on agar plates, so purification to axenic culture was achieved through serial dilution. Purity was verified microscopically (absence of cells lacking autofluorescence), by enriching for heterotrophs using peptone-yeast extract–amended media, by analysis of 16S rDNA profiles of cultures (denaturing gradient gel electrophoresis of the v3 region), and by cloning and sequencing libraries of nearly full-length (9F/1492R) PCR amplicons.

Growth media and culture conditions—The growth medium used for all experiments contained the following (per liter): 100 mg $MgSO_4 \cdot 7H_20$, 50 mg $CaCl_2 \cdot 2H_2O$, 25 mg K_2HPO_4, and 420 mg $NaNO_3$. Sodium carbonate was added (10 mmol L^{-1} final concentration), and pH was adjusted to 8.0. NaCl was used to increase salinity, depending on the experiment and the growth requirements of each strain. Vitamin, trace metal, and iron stock solutions were added to this basal medium (0.5 mL of each per 1 L). The vitamin stock solution contained the following (per liter): 5 mg folic acid (B_9), 5 mg cyanocobalamin (B_{12}), 5 mg biotin, 40 mg Ca-pantothenate (B_5), 70 mg thiamin (B_1), 40 mg nicotinic acid (niacin), 40 mg *p*-aminobenzoic acid, and 20 mg pyridoxolium hydrochloride. The trace metal stock solution contained the following (per liter): 1,000 mg H_3BO_4, 450 mg $MnCl_2 \cdot 4\ H_2O$, 50 mg $ZnSO_4 \cdot 7\ H_2O$, 20 mg $Na_2MoO_4 \cdot 2H_2O$, 20 mg $Co(NO_3)_2 \cdot 6H_2O$, and 5 mg Na_2SeO_4. The iron stock solution contained the following (per 200 mL): 300 mg $FeCl_3$, 500 mg sodium citrate, and 100 mg EDTA. Nutrients, vitamins, trace metals, sugars, neutralized sulfide, and other amendments were added to autoclaved salts solutions as filter-sterilized solutions. Agar plates were prepared with purified agar at 0.8% w/v. Unless otherwise noted, all experiments were run aerobically in a temperature-controlled incubator (20°C) with constant light from cool-white fluorescent tubes (40 μmol quanta $m^{-2}\ s^{-1}$). Anoxic media was prepared aerobically and then placed in an anaerobic chamber for 2 days prior to use. Liquid media was then filter-sterilized and dispensed into sterile serum bottles that were inoculated, closed with sterile butyl rubber stoppers, and placed in the appropriate incubator. Growth rate as a function of irradiance was determined by incubating cultures at varying distances from a constant source of light from cool-white fluorescent tubes. Temperature response experiments were conducted by setting incubators at different temperatures at the same irradiance.

Instantaneous growth rates (μ) were calculated from the logarithmic change of in vivo fluorescence measured once per day with a Turner Designs fluorometer. The room was darkened during measurements made for irradiance response experiments. Typically, growth was followed for 1–4 weeks, but under certain conditions (high salinity, low light), growth was slow and was monitored for up to 8 weeks. All cultures were acclimated to the test conditions for at least three transfers before measurements were made.

Organotrophic growth was tested by wrapping culture vessels in aluminum foil, placing them in a sealed box, and then placing the box in a dark incubator. Basal media was supplemented with either sucrose, fructose, glucose, yeast extract, or peptone, and growth was monitored for 1 month by epifluorescence microscopy. The isolate's capability for light-activated, dark organotrophic growth was tested by placing cultures in the light for 20 minutes per day. Sulfide tolerance was tested several times with and without sulfide-acclimated cells. Basal media was amended with 0–1 mmol L^{-1} total sulfide (neutralized) and adjusted to a pH of 8.0 for sulfide tolerance experiments. HEPES was tested as a buffer, but strain MLCB does not grow well at neutral pH. Culture growth was monitored by epifluorescence microscopy, and sulfide concentrations were checked periodically.

Pigment analysis—Absorption spectra were obtained in vivo using a Shimadzu UV160U spectrophotometer as previously described (Garcia-Pichel and Castenholz 1991). In vivo fluorescence spectra of phycoerythrobilin (PEB; $\lambda_{AbsMax} \cong$ 540–570 nm) and phycourobilin (PUB; $\lambda_{AbsMax} \cong$ 495–500 nm) were obtained using a Shimadzu RF-5000 spectrofluorometer as follows: excitation illumination ranged from 450 to 580 nm with the emission monochromator set at 560 or 588 nm, respectively, and a 3-nm band pass. Complementary chromatic adaptation potential was determined as previously described (Tandeau De Marsac and Houmard 1988). Morphology and ultrastructure of the isolate were determined by transmission electron microscopy (TEM) performed on a JEOL 100 CX TEM as previously described (Stanier 1988).

Sequencing and phylogenetic analysis—Nucleotide sequences for the 16S rRNA gene, the 16S–23S rRNA gene internal transcribed spacer (ITS) region, form I ribulose-1,5-bisphosphate carboxylase (*cbbL*), and *nifH* genes were obtained as described previously (Rocap et al. 2002; Giri et al. 2004; Steward et al. 2004). All sequences determined in this study have been deposited in GenBank under accession numbers EF630354, EF630355, and AY382480. The 16S rRNA gene reference sequences used in this study were obtained from the Ribosomal Database Project (Cole et al. 2007). All available cyanobacterial sequences (2,125 total, >1,200 bp) were downloaded and imported into ARB (Ludwig et al. 2004). Neighbor-joining trees were constructed, and sequences belonging to the Pcy lineage were exported to a new ARB database. Within ARB, ClustalW (automatic mode) was used to realign the Pcy sequences, the alignment was checked manually, missing data were denoted with question marks, and reference sequences were selected and then exported to Nexus file format.

ITS sequences were downloaded from GenBank and aligned in ClustalX (Chenna et al. 2003). Sequences from highly similar strains were aligned among each other first, then all sequences were divided into five sections bracketed by conserved regions of the 16S or 23S rRNA genes or by tRNAs or stem-loop structures. Each section was then aligned separately. The alignments were checked manually for misalignments and then exported into Nexus file format, with and without the inclusion of tRNAs. The software program MODELTEST (Posada and Crandall 1998) was used to determine the best-fit model of nucleotide substitution for the data. MODELTEST identified the Tamura-Nei (TrN) model as the best fit for the 16S rRNA gene data and the Hasegawa (HKY) model as the best fit for the ITS data, both with and without tRNAs. Parsimony, distance, and maximum likelihood criteria were used to construct phylogenetic trees in the programs PAUP and ARB (Swofford 2002; Ludwig et al. 2004). ARB is better suited for manipulating large data sets and for creating trees, where PAUP provides more features and a robust set of tools. We created numerous trees using various subsets of the data to test the robustness of tree topology and to compare the topology of 16S rRNA gene and ITS trees.

Cyanobium strains used in comparative studies were obtained from the Pasteur Culture Collection (PCC 6307, PCC 7001, PCC 9005), the Netherlands Institute of Ecology, Centre for Estuarine and Marine Ecology (BO 8807, CCY 011, CCY 9504, CCY 9505, LBP1), and the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP836).

Results

Pcy distributions—Interrogation of Mono Lake samples using general bacterial primers (Hoeft et al. 2002; Humayoun et al. 2003) and form I ribulose-1,5-bisphosphate carboxylase (*cbbL*) primers (Giri et al. 2004) indicated the presence of only one Pcy genotype in the anoxic waters of Mono Lake. The sequences obtained were identical (>99%) to sequences from strain MLCB, and all cell counts from the water column were of a PE-containing Pcy. Thus, we are confident that the epifluorescence microscopy counts are of the same organism that was isolated into pure culture; however, given the limited resolution of our sequence data, we cannot rule out the existence of other strains in the lake. To preserve the distinction between data from epifluorescence counts of field samples and data obtained from laboratory cultures of an isolate from the field population, we use "Pcy" to refer to field data and "strain MLCB" or "the isolate" to refer to the cultured organism.

Vertical profiles of Pcy abundance (Fig. 2) show that cells were always more abundant in bottom water than in the surface mixed layer and were detected in the surface mixed layer only in the autumn and early winter. Pcy were also enumerated in samples taken from a sediment core collected in August 2003, where it was found at a concentration of 1.2×10^6 cells mg dry wt^{-1} in the 0–4-cm depth horizon (data not shown).

Integrating concentration data over depth to compare photic (0–25 m) versus the aphotic (25–40 m) zone populations indicated that the Pcy population was always greatest in the aphotic zone and that this population decreased through winter and spring until late summer (Fig 3). Lakewide inventories of Pcy cells in the two layers were calculated using the hypsographic curve and our abundance data (and assuming uniform horizontal dis-

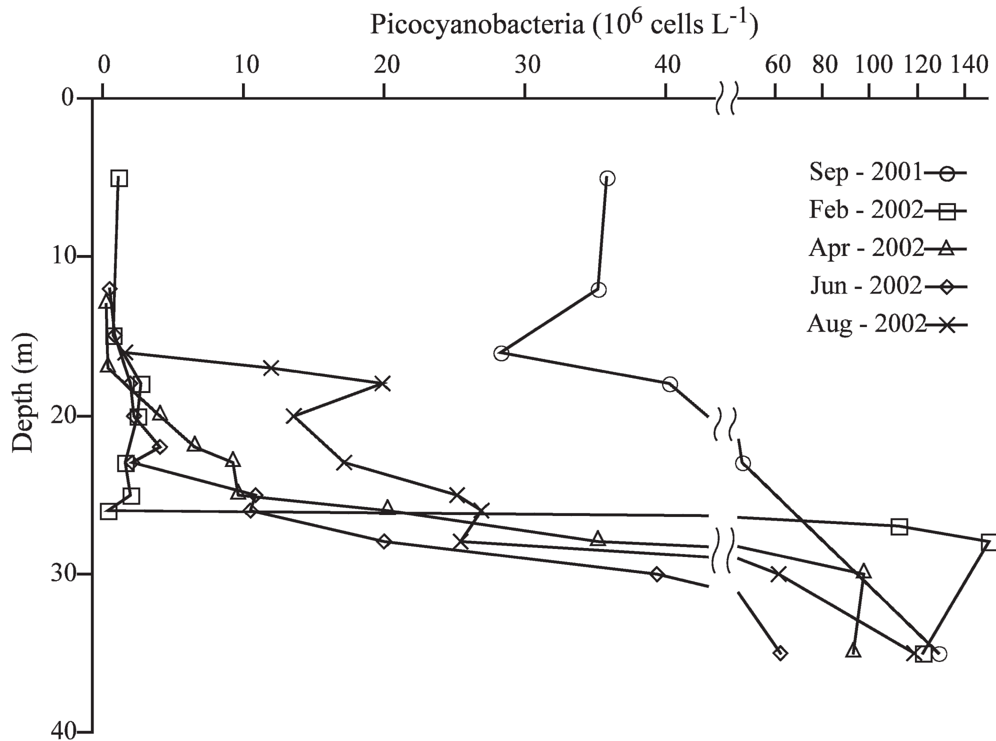

Fig. 2. Picocyanobacteria concentration (cells L^{-1}) over an annual cycle (September 2001–August 2002). Note break and change in x-axis scale.

tributions). This analysis indicated that the Pcy inventory was greater in the photic zone than in the aphotic zone only in autumn (data not shown). The lake, which had been meromictic for 8 years, mixed completely over the winter of 2003–2004. The lakewide inventory of Pcy in April 2004 was much lower (~90%) than in the two previous springs, primarily as a result of a large decrease in the aphotic zone population (Fig. 4).

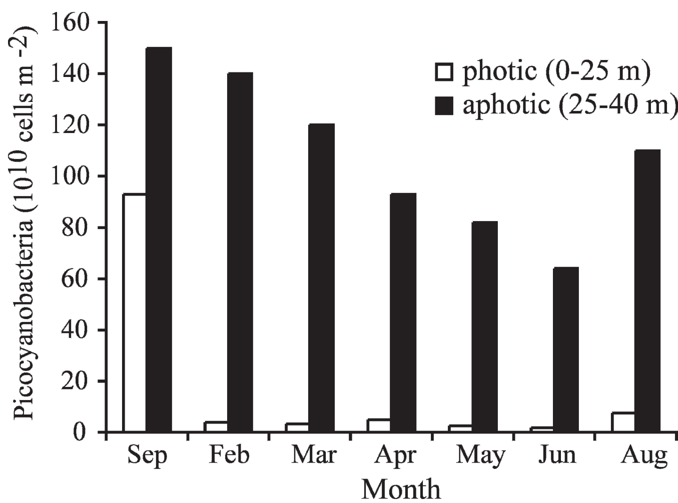

Fig. 3. Picocyanobacteria inventory (cells m^{-2}) over an annual cycle (September 2001–August 2002) integrated over the photic (open bars, 0–25 m) and aphotic (black bars, 25–40 m) zones of Mono Lake.

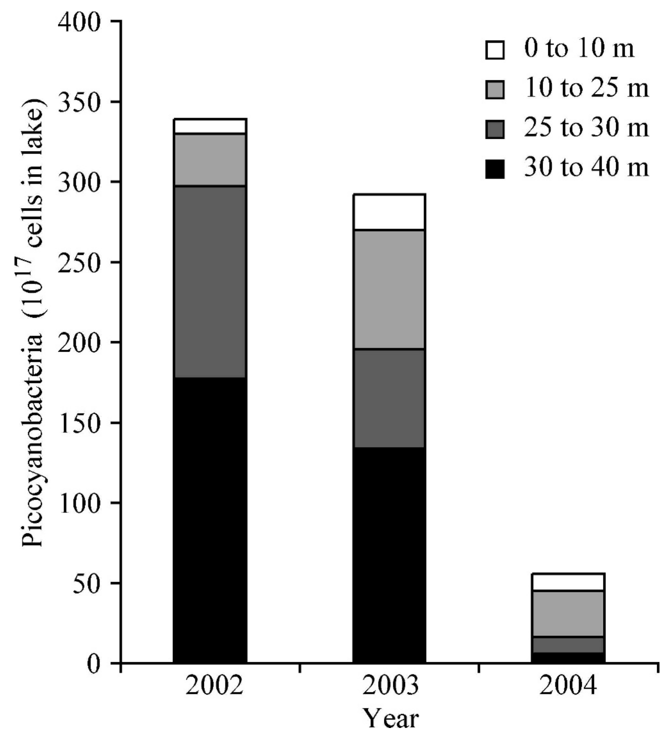

Fig. 4. Lakewide inventories of picocyanobacteria (10^{17} cells) in different depth strata during three consecutive winters: no shading, 0–10 m; light gray shading, 10–25 m; dark gray shading, 25–30 m; black shading, 30–40 m.

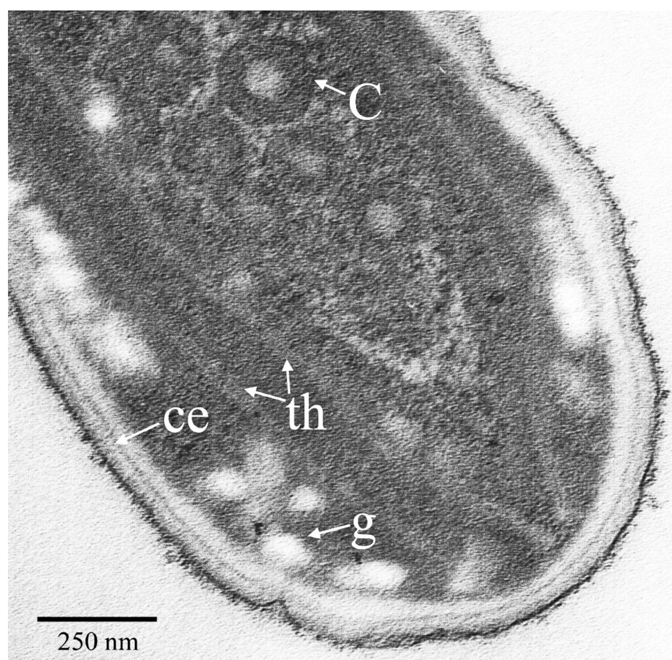

Fig. 5. Transmission electron micrograph (TEM) of strain MLCB. C, carboxysomes; ce, cell envelope; th, thylakoids; g, glycogen deposits.

Cell morphology—Examination of field and cultured cells by epifluorescence microscopy and TEM (Fig. 5) show strain MLCB to be 2 × 1 μm and to divide by binary fission. Cells are capable of elongated growth in culture, resulting in a long (to 10 μm) cell. This cell morphology occurs at all salinities and temperatures but is only found in stationary phase. We have occasionally seen elongated Pcy cells in Mono Lake samples. Aggregates of Pcy cells were found in field samples, occurring rarely (mainly in surface waters) and consisting of 5–20 cells.

Pigments—In vivo absorption and fluorescence spectra of exponentially growing cells in freshwater medium indicated phycoerythrobilin as the dominant chromophore (data not shown). Strain MLCB did not contain PUB (data not shown) and was not capable of complementary chromatic adaptation (CCA); it grew well in green light and slightly slower in red light while maintaining its PE-to-PC ratio (data not shown). This finding is consistent with other *Cyanobium* isolates, which are not capable of CCA and do not contain PUB. At low light intensities (<75 μmol quanta m^{-2} s^{-1}) and low salinities (<5%), cells retained a dark red color. At high light intensities (>100 μmol quanta m^{-2} s^{-1}) and high salinities (>8%), cells became yellow or light brown, typical of stressed cells.

Salinity tolerance—Strain MLCB is euryhaline, growing from 0% to 10% salinity with a maximum specific growth rate of 0.45 d^{-1} at 3% and a minimum (measurable) growth rate of 0.15 d^{-1} at 8% (Fig. 6A). We compared the salinity tolerances of seven *Cyanobium* isolates to strain MLCB (Fig. 7). Strains isolated from salt waters showed the highest growth rates at 1.5% to 3% salinity. Strains

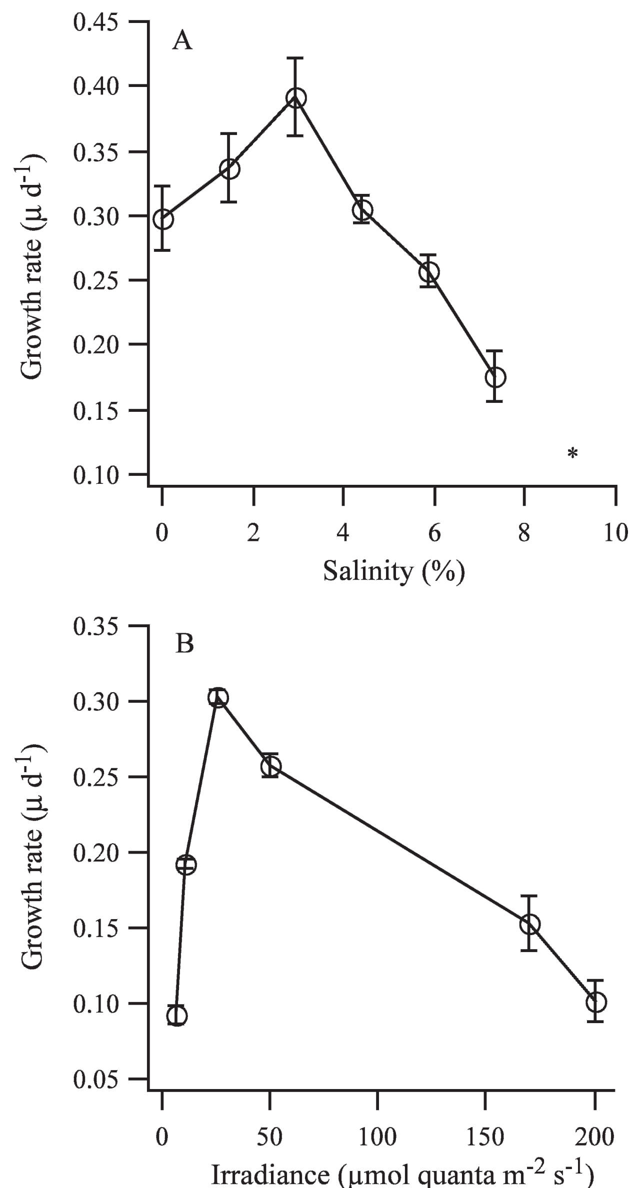

Fig. 6. Effect of (A) salinity and (B) light intensity on the specific growth rate μ (d^{-1}) of strain MLCB. Data are means ±SD (n = 3). Asterisk (*) indicates visible growth that was not measurable.

from fresh water grew best when no salt was present. We also tested CCMP836 (coastal *Synechococcus* strain WH 8007) and determined that it was not able to grow in freshwater (data not shown).

Growth rate—Strain MLCB grew at all light intensities tested (Fig. 6B) with a maximum specific growth rate of 0.45 d^{-1} at 40 μmol quanta m^{-2} s^{-1}, decreasing to 0.15 d^{-1} at 200 μmol quanta m^{-2} s^{-1}. Maximal growth rates occur at ~20°C (data not shown; 4°C, 20°C, and 27°C were tested). Growth rates decreased rapidly at temperatures above 27°C, and cells were noticeably stressed (pigment loss). Strain MLCB can perform oxygenic photosynthesis in up to ~500 μmol L^{-1} total sulfide at

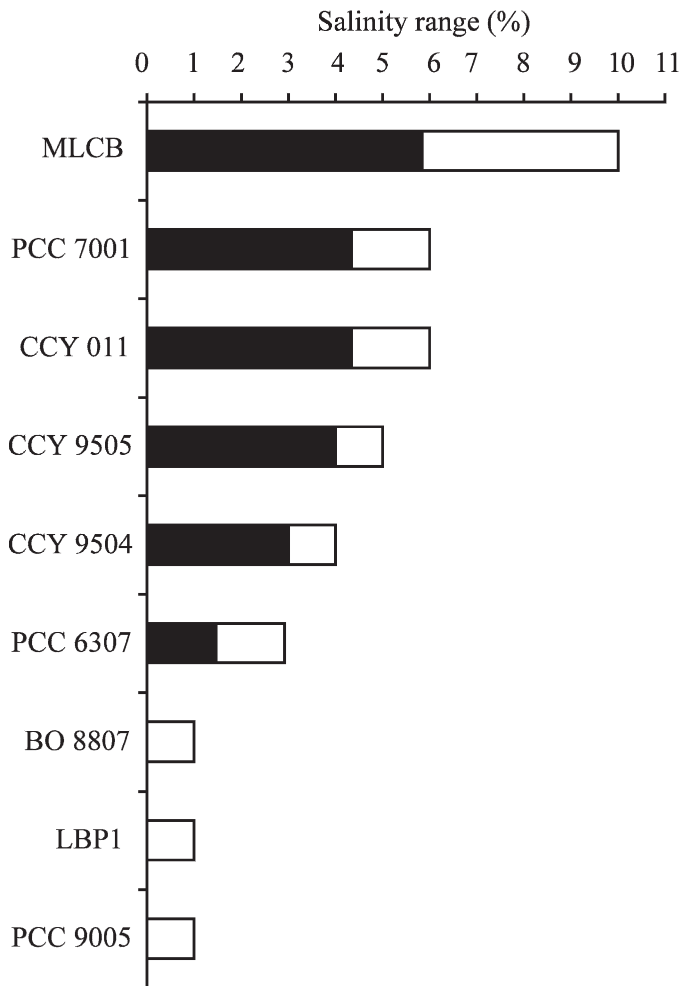

Fig. 7. Comparison of the salinity responses of a selection of *Cyanobium* strains. Optimal growth (black bars), defined as salinities where specific growth rates were above 0.2 d^{-1}; suboptimal growth (white bars) indicates salinities where the specific growth rate was positive but <0.2 d^{-1}.

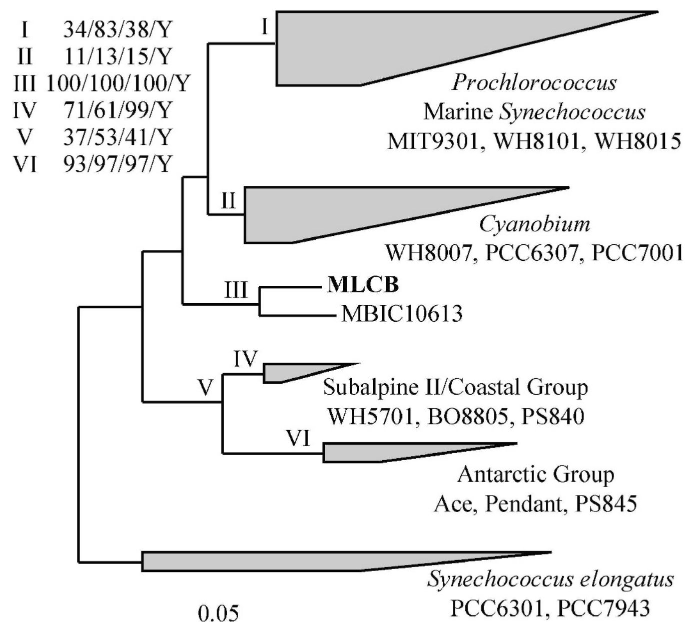

Fig. 8. Phylogenetic inference of the picocyanobacteria clade sensu Urbach et al. (1998) based on 16S rDNA sequences. Distance bootstrap values (1,000 replicates) are given at the nodes and were determined by an analysis of three data sets as described in the text. First value, dendrogram, and scale bar were generated in ARB for 279 taxa, 1,623 positions, and JC correction. The second value is from PAUP for 87 taxa, 1,623 positions, and TrN correction. The third value is from PAUP for 86 taxa, 1,236 positions, and TrN correction. The letter Y indicates that the node was present in a maximum likelihood analysis of the third data set. Representative strains for the clades are given. Scale bar indicates evolutionary distance (substitutions per bp).

a pH of 8.0, but growth was inhibited above this concentration. Anoxygenic photosynthesis was not detected.

Chemotrophy—Strain MLCB was not capable of dark chemoorganotrophic growth, either aerobically or anaerobically, on glucose, fructose, sucrose, yeast, or peptone as substrates or via a light-activated pathway. However, cells placed in the dark without a carbon source turned from dark red to pale green/yellow, indicating the degradation of phycobilins and chlorophyll, while cells given sucrose remained dark red (data not shown). We did not test for mixotrophic growth.

Phylogenetic analysis—Sequences for the *cbbL* gene from strain MLCB (AY382479) and from environmental samples containing the Mono Lake Pcy have been reported elsewhere (Giri et al. 2004). Strain MLCB apparently does not contain a *nifH* gene, as we were unable to obtain PCR products from it using standard primers. Phylogenetically, strain MLCB grouped with the picophytoplankton clade of cyanobacteria (Urbach et al. 1998), thus being related to *Prochlorococcus*, marine *Synechococcus*, and *Cyanobium* type strains. Analysis of 16S rDNA sequences indicates that the isolate was most closely related to strain MBIC 10613 (AB183569), isolated from coastal waters near Kagawa, Japan.

An exhaustive phylogenetic analysis of the 16S rRNA gene revealed that strain MLCB does not group strongly with either the marine Pcy or the *Cyanobium* (Fig. 8). Because of the high similarity of 16S rRNA gene sequences among the Pcy, phylogenetic analysis is sensitive to the protocol used, the number of strains used, and the taxa selected. Figure 8 presents a consensus of our analysis and includes bootstrap values derived from three separate data sets. The first data set was analyzed by ARB and contained 296 taxa and 1,601 positions, used the Jukes-Cantor (JC) correction, and contained 1,000 replicates (the dendrogram shown in Fig. 8). The second data set was analyzed by PAUP, contained 90 taxa and 1,601 positions, used the TrN correction, and contained 1,000 replicates. The third data set was analyzed by PAUP, contained 90 taxa and 1,129 positions (no missing data), used the TrN correction, and contained 1,000 replicates. In general, the three data sets yielded the same tree topology. Notable differences include a closer association between MLCB and the marine

Synechococcus/*Prochlorococcus* cluster with data set 2 and a closer association of MLCB and node V with the *Cyanobium* for data set 3 versus the other analyses.

16S rDNA trees of coastal and freshwater Pcy (constructed without *Prochlorococcus* and marine *Synechococcus* sequences) contained at least eight distinct clades with high bootstrap support (Fig. 9). This tree topology is in general agreement with previous studies (Crosbie et al. 2003; Ernst et al. 2003), with differences occurring mostly at nodes exhibiting low bootstrap support and due to the addition of new sequences.

The 16S-23S ITS region from strain MLCB was 911 bp long with a G+C of 54.9%. The topology of our ITS trees generally agreed with those of a previous study (Ernst et al. 2003). MLCB grouped strongly with the *Cyanobium* in ITS trees (Fig. 10), reflecting the similarity in ITS sequence length between MLCB and *Cyanobium*. ITS alignments containing all the Pcy (i.e., including the marine Pcy) were ambiguous, in part because there are large differences in sequence length as well as in sequence content within this larger data set. There was a significant difference in the position of strain WH 8007 and its relatives in trees from our study versus Chen et al. (2006). Our 16S and ITS data clearly show WH 8007 grouping strongly with the *Cyanobium*, where Chen et al. (2006) placed it (58% bootstrap support) within the marine *Synechococcus*.

Discussion

Pcy population dynamics—The highest concentrations of Pcy in Mono Lake were always found in the aphotic, anaerobic bottom water (Fig. 2). Studies of other lakes have revealed the presence of Pcy populations at depths below 0.1% surface irradiance (Craig 1987; Detmer et al. 1993; Malinsky-Rushansky et al. 1997; Padisak et al. 1997), but they have not reported populations that are 2 to 10 times greater at depth than in the photic zone, such as we found in Mono Lake. Three years of data taken when the lake was meromictic show that Pcy bloomed in surface waters in late summer and early autumn while remaining practically undetectable in photic zone waters from late winter through the summer. These seasonal growth patterns are not uncommon for Pcy (Stockner et al. 2000) and lead us to propose the following conceptual model to explain the seasonal distribution of the Mono Lake Pcy ecotype.

Pcy blooms in the epilimnion in late summer and early autumn when surface waters are warm ($\sim$20°C), populations of microzooplankton have been reduced by *Artemia* grazing, and nutrient availability has increased in response to the onset of the fall overturn. As mixing continues through autumn and winter, nutrient concentrations increase, and water temperatures decrease (to $\sim$2°C), slowing the growth of Pcy. Populations of eukaryotic algae and microzooplankton increase in the absence of grazing by *Artemia*. As light availability increases in spring, nutrients are plentiful, water temperatures are low, and eukaryotic algae bloom, while Pcy concentrations decrease because of microzooplankton grazing and low, temperature-limited growth rates. By late spring and early summer, Pcy are barely detectable in surface waters, but concentrations remain high in anoxic waters below the chemocline. The pulse of sinking phytoplankton that accompanies thermal stratification, nutrient depletion, and increased *Artemia* grazing in early summer may strip the upper water column of remaining Pcy, depositing them in bottom water or at the sediment surface. High light, low nutrients, and heavy *Artemia* grazing prevent recovery of Pcy populations as the epilimnion warms during summer.

Drought and water diversions can trigger the complete overturn (holomixis) of Mono Lake (Jellison and Melack 1993*b*). Holomixis occurred in late October to early December 2003, for the first time since 1995. In November 2003, concentrations of Pcy in the photic zone were high, consistent with the autumn bloom of previous years. However, we noted a significant decrease in Pcy populations by April 2004 (Fig. 4). The decrease was most pronounced in the aphotic zone, while abundance in the photic zone was comparable to previous years. The lakewide inventory of Pcy cells was much lower in April 2004 than during the previous two springs, suggesting that the decreased bottom-water population was not simply due to mixing. We conclude that turnover appears to eliminate the bottom-water Pcy population, perhaps by mixing them into the surface layer, where they are lost because of grazing, competition, UV sensitivity, phage lysis induced by drastically altered growth conditions, and so on. Mono Lake also mixed during the winter of 2004–2005, and, although we do not have abundance data from the preceding autumn or the following spring, we found the typical vertical distribution in September 2005, with 6.0×10^7 cells L^{-1} in the photic zone and 1.5×10^8 cells L^{-1} in bottom waters. This suggests that even though winter overturn eliminates the bottom-water population, these populations are restored by the following autumn. We have no definitive explanation for the high concentrations of Pcy in the aphotic zone. We offer the following hypotheses to explain the seasonal accumulation of Pcy cells at depth.

Sinking of cells produced in the photic zone is an obvious possible explanation for elevated Pcy concentrations at depth. Although extremely small and with a negligible sinking velocity, Pcy are known to sink, possibly by attaching to detritus or fecal pellets, thus increasing their sedimentation rate (Lochte and Turley 1988; Simon et al. 2002). Sedimentation rates are also enhanced by aggregation, and we have observed Pcy aggregates in the upper water column. We have observed clumped and elongated cells in stationary phase cultures of strain MLCB. Since the upper water column of Mono Lake is nitrogen limited during summer, perhaps nutrient limitation causes clumping, resulting in higher sinking rates or making cells susceptible to grazing by *Artemia*.

Why single cells would accumulate in deep water (rather than sink to the sediment surface in aggregates) is unclear. Certainly they do sink to the sediment surface, as we were able to enumerate them in samples of surficial sediments from station 6 by epifluorescence microscopy. An analysis of the distribution of rRNA genes in sediment cores from station 6 (Hollibaugh et al. unpubl. data) indicates that DNA from the Pcy is abundant at distinct horizons in the

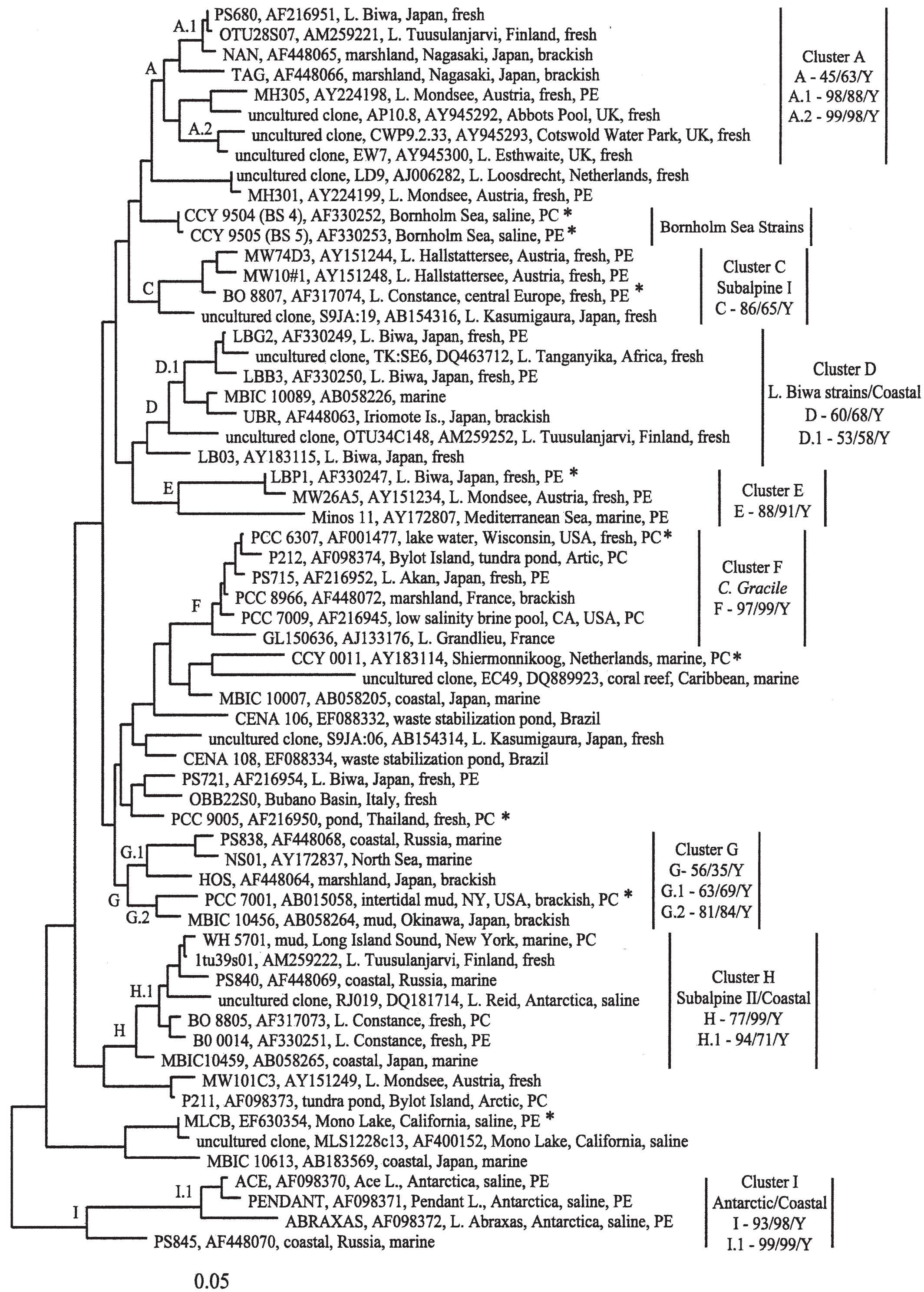

Fig. 9. Habitat and phylogenetic inference of select picocyanobacteria based on 16S rDNA sequences. Distance bootstrap trees were created using an analysis of two data sets. Clades with high bootstrap support that were present in all trees and that contained three or more distinct taxa are noted as clusters delineated by vertical bars bracketing the cluster identifier. Bootstrap values (%) for nodes indicated by letters are shown under the cluster designation. The first value, dendrogram, and scale bar were generated in ARB for 1,623 positions and JC correction. The second value is from PAUP for 1,331 positions and TrN correction. The letter Y indicates that the node was present in a maximum likelihood analysis of the second data set. Terminal branches display the strain information: GenBank accession number, source, water type, and dominant phycobilin. Asterisks (*) indicate strains used for salinity tolerance experiments. Scale bar indicates evolutionary distance (substitutions per bp). Physiological information on the strains came from the following sources: Crosbie et al. (2003); Ernst et al. (2003); Pasteur Culture Collection (France); GenBank (NCBI); and Marine Biotechnology Institute Culture collection (Japan).

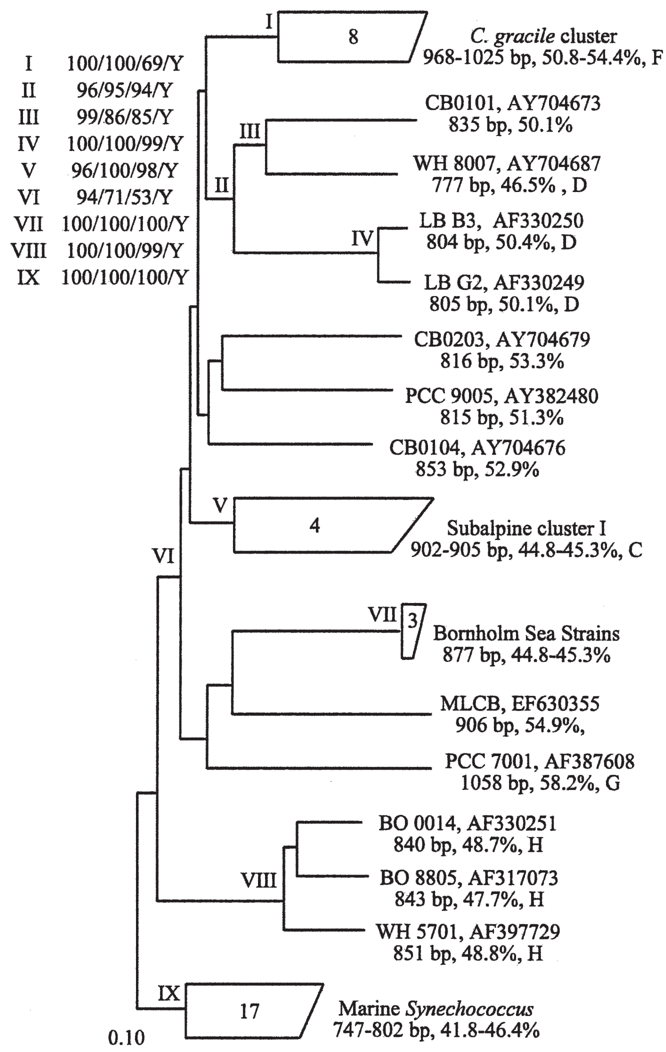

Fig. 10. Phylogenetic inference of the picocyanobacteria based on the 16S–23S ITS region. Distance bootstrap values (1,000 replicates) are given for nodes displaying >50% support. The first value, dendrogram, and scale bar were generated in ARB using the entire spacer region (1,212 positions used in the analysis, including tRNAs) with the JC model. The second value is from PAUP using the entire spacer region with the TrN correction. The third value is from PAUP using a data set with tRNAs removed (1,061 positions included in the analysis) with TrN correction. The letter Y indicates that the node was present in a maximum likelihood analysis of the third data set. Labels within boxes indicate the number of sequences in that clade. Lengths (bp, including tRNAs) and G+C % content of the regions used in the analysis are given along with the cluster designation from Fig. 4. Scale bar indicates evolutionary distance (substitutions per bp).

sediment core corresponding to 10 and 50 years before the present, suggesting massive deposition events. However, in order for cells sinking in aggregates to accumulate in bottom water, a mechanism for dispersing the aggregates would have to exist. Once dispersed, the slightly greater density of bottom water and (possibly) increased viscosity resulting from observed elevated dissolved organic carbon concentrations in bottom water (Hollibaugh unpubl. data) would slow sinking.

Individual Pcy cells sinking at a constant rate could also attain elevated concentrations in the hypolimnion if epilimnion concentrations exceeded the monimolimnion concentrations at some time prior to our sampling (i.e., a sinking bloom). Analysis of our data indicates a slow loss of cells from bottom waters and supports their sudden replenishment at the end of the summer, when Pcy can also be found in the epilimnion. This suggests that growth in surface waters during the autumn bloom is at a least partly responsible for replacing the monimolimnion population. However, our data (Fig. 2) failed to indicate a time when the epilimnion Pcy population was high enough to account for monimolimnion populations by simply sinking.

Littoral transport combined with sediment resuspension may also provide a mechanism for increasing Pcy abundance in the monimolimnion. Because the lake basin is relatively saucer shaped, the lakewide inventory of cells in the epilimnion during the autumn is greater than the inventory in the monimolimnion (data not shown), despite the difference in concentration. This implies that it is possible for sedimentation coupled with lateral mixing or transport to supply enough cells to the monimolimnion to account for the observed populations. Mono Lake experiences strong (6–8 m s^{-1}) diurnal winds for much of the year, setting up cyclonic flows and boundary mixing, which could support horizontal transport into the study site (MacIntyre et al. 1999).

Strain MLCB physiology—Cell structure and physiology are also factors that could contribute to survival of Pcy populations in the monimolimnion. Some cyanobacteria, particularly filamentous and benthic cyanobacteria, are capable of dark chemoorganotrophic growth on exogenous carbon sources using the oxidative pentose-phosphate cycle (Pelroy and Bassham 1972). Strain MLCB was not capable of chemoorganotrophic growth in continuous darkness or with brief exposures to light on any of the substrates we tested. However, strain MLCB appears to be capable of using sucrose for cell maintenance, particularly of pigment composition. It is of course possible that unknown growth factors or growth conditions are required to stimulate chemoorganotrophic growth or that strain MLCB is capable of using some combination of substrates that we have not tested, such as reduced sulfur compounds. Although light levels below ~25-m depth (<0.0004 μmol quanta m^{-2} s^{-1}) do not seem to be high enough to support growth solely by photoautotrophy, its possible that enough photons reach the monimolimnion to supplement chemotrophic growth.

Finally, selective predation could be responsible for contrasting distributions of Pcy and eukaryotic algae. Grazing studies at Mono Lake have been limited to surface waters and have focused on *Artemia monica* grazing on eukaryotic algae (Conte et al. 1988). Protozoan grazing has not been examined, nor has grazing of any sort in the anoxic waters, in part because *Artemia* avoid hypoxic and anoxic water.

Salinity tolerance is a central physiological trait that separates strain MLCB from the eukaryotic algae of Mono Lake and from other Pcy. Strain MLCB is able to survive

at salinities up to 10%. *Picocystis* grows at salinities up to 26% (Roesler et al. 2002), and mesocosm experiments with benthic diatoms from Mono Lake showed activity at 15% (Herbst and Blinn 1998). These studies also indicated a decrease in algal species diversity as salinity exceeded 5%. Strain MLCB's lower salinity tolerance could hamper its ability to compete with eukaryotic algae at low stands of the lake. In addition, the increase in Mono Lake's salinity over the past 50 years of net evaporation could also be responsible for the fact that the lake appears to contain only one dominant Pcy ecotype.

Relationship between phylogeny and physiology—There does not appear to be a correlation between Pcy salinity ecotype and 16S rDNA phylogeny, as freshwater strains group repeatedly with marine or brackish strains (Fig. 9). Furthermore, as shown by strain PCC 6307, isolation from a freshwater environment does not preclude a strain from being halotolerant, nor are coastal strains like WH 8007 that cluster with freshwater strains necessarily capable of growth in freshwater. Based on this information, it appears that the ability to withstand elevated salinity has been lost and possibly modified repeatedly in the evolution of the Pcy.

A final point we would like to make is that our comparisons of phylogenetic relationships among all Pcy based on 16S rRNA genes versus the ITS region suggest that use of the ITS region at this phylogenetic scale is problematic and can be misleading. This is due primarily to the large differences in ITS sequence length among the Pcy. For example, PCC 7001 has an ITS length similar to strain MLCB and groups closely with strain MLCB in ITS-based phylogenetic analysis; however, the similarity of their ITS sequences (when conserved regions in tRNAs and stem-loop structures are excluded) is low. In contrast to Chen et al. (2006), we found that the ITS region was not a good marker for examining phylogeny across the Pcy. Our results suggest that phylogenetic analyses based on ITS sequences should be reserved for investigations of closely related clades and ecotypes.

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