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Physiological and gene expression responses to nitrogen regimes and temperatures in *Mastigocladus* sp. strain CHP1, a predominant thermotolerant cyanobacterium of hot springs

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ABSTRACT

Cyanobacteria are widely distributed primary producers with significant implications for the global biogeochemical cycles of carbon and nitrogen. Diazotrophic cyanobacteria of subsection V (Order Stigonematales) are particularly ubiquitous in photoautotrophic microbial mats of hot springs. The Stigonematal cyanobacterium strain CHP1 isolated from the Porcelana hot spring (Chile) was one of the major contributors of the new nitrogen through nitrogen fixation. Further morphological and genetic characterization verified that the strain CHP1 belongs to Stigonematales, and it formed a separate clade together with other thermophiles of the genera *Fischerella* and *Mastigocladus*. Strain CHP1 fixed maximum N_2 in the light, independent of the temperature range. At 50 °C *nif*H gene transcripts showed high expression during the light period, whereas the *nif*H gene expression at 45 °C was arrhythmic. The strain displayed a high affinity for nitrate and a low tolerance for high ammonium concentrations, whereas the *nar*B and *gln*A genes showed higher expression in light and at the beginning of the dark phase. It is proposed that *Mastigocladus* sp. strain CHP1 would represent a good model for the study of subsection V thermophilic cyanobacteria, and for understanding the adaptations of these photoautotrophic organisms inhabiting microbial mats in hot springs globally.

Introduction

Cyanobacteria are microorganisms responsible for the oxygenation of the Earth's atmosphere [9], thereby structuring the biosphere for the evolution of plants and animals. They have also successfully colonized almost all illuminated environments and numerous plant hosts [73,69]. Furthermore, because they are important primary producers in many environments, they are also responsible for a substantial proportion of the new nitrogen fixed into oceans [10], soils [6,17], lakes [64], and hot springs [11]. Cyanobacteria also exhibit remarkable morphological diversity. Their members are subdivided into subsections I–V [49], the last two of which are supported by 16S rRNA gene phylogeny [75]. Recently, Dagan et al. [15] showed that up to 20% of the eukaryotic nuclear genes are of cyanobacterial origin, especially from subsections IV and V (plastid ancestors). Although our understanding of multicellularity in subsection IV has deepened considerably due to studies of *Anabaena* sp. PCC7120 as a model organism [21], little is known about the cyanobacteria of subsection V.

Members of the order Stigonematales (subsection V), which are among the most complex types of cyanobacteria, are commonly found in a variety of terrestrial environments [62,52,59,17], endolithic cavities [13,24], and microbial mats from hot spring ecosystems [34,51,8,61,66,42,36,2] and are considered cosmopolitan organisms with relevant importance as phototrophs and diazotrophic cyanobacteria [11].

There are six genera from subsection V with partially sequenced genomes, but only one has been completed (*Fischerella* sp. NIES-

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3754; [25]). Among this subsection, thermophilic members within the genera *Chlorogloeopsis*, *Fischerella* and *Mastigocladus* have been reported [51,19,26]. However, Kaštovský and Johansen [29] suggested that the thermophilic character separates the *Fischerella* and *Mastigocladus* genera, and that all thermal members should be assigned to the *Mastigocladus* genus [29]. Within this genus, the species *Mastigocladus laminosus* has been reported as a major component of epilithic microbial mats in Yellowstone National Park (YNP, USA) [8,42,43,72].

In filamentous cyanobacteria from subsections IV (Nostocales) and V (Stigonematales), growth under nitrogen depletion causes changes in the ultrastructure of photosynthetic vegetative cells, with distinct patterns of reorganization of subcellular components (e.g. thylakoid membranes, cell walls) [63,32,57], eventually leading to the differentiation of heterocysts, which are specialized nitrogen-fixing cells [37]. Most N_2-fixing cyanobacteria are mesophilic and show optimum N_2-fixing ability in the moderate temperature range of 20–25 °C [27]. At high temperature, various metabolic processes, including N_2-fixing ability, might be adversely affected, but information concerning N_2-fixing and assimilation is scarce in the case of filamentous thermophilic cyanobacteria.

Members of the *Stigonematales* that live in hot springs, specifically *Mastigocladus* sp., have been found to be primarily responsible for the nitrogen fixed at temperatures between 28 and 60 °C [74,66,67,2]. Consistent with this finding, and besides the fact that *Mastigocladus* spp. have been found even at 63 °C [26], 60 °C is the highest temperature recorded to date for the activity of these N_2-fixing filamentous cyanobacteria [2], and represents a proposed upper temperature limit for their in situ nitrogen fixation. Furthermore, Alcamán et al. [2] demonstrated by in situ assays that maximum *nif*H gene transcript levels were associated with the *Mastigocladus* genus, which correlated with the higher nitrogenase activity observed. These and other features of *Mastigocladus* spp. make them ecologically important as a component of microbial mats that are primary producers in neutral-to-alkaline thermal springs [12].

In cyanobacteria, ammonium is usually the preferred inorganic nitrogen source due to its lower energy requirement. Ammonium is generated from the reduction of nitrate, through nitrogen fixation or directly through incorporation via the permease transport system (Amt) and is subsequently incorporated into amino acids and proteins through the GS;*gln*A–GOGAT;*glt*S route [44]. On the other hand, nitrate assimilation involves the transport system encoded by the *nrt*ABCD genes, and the subsequent reduction into ammonium is catalyzed by nitrate reductase (NR; *nar*B) and nitrite reductase (NiR; *nir*A) [20,53,23].

Under controlled conditions, the thermophilic stigonematal *M. laminosus* shows typical phenotypic (differentiation of heterocysts) and genetic responses to the lack of combined nitrogen, including increased expression of the *nif*H gene, which encodes one of the subunits of the nitrogenase enzyme [63,30]. Furthermore, in a thermotolerant *M. laminosus* species isolated from the Jakrem hot spring in Meghalaya, India, nitrogenase activity was inhibited in the presence of combined nitrogen sources, such as nitrate (NO_3^-), ammonium (NH_4^+) and amino acids [30], which is similar to findings for other non-thermal heterocyst-forming cyanobacteria (e.g. *Anabaena* sp. PCC7120; subsection IV) [39,70,71,38,20]. This thermophilic *M. laminosus* species is able to assimilate efficiently NO_3^- and NH_4^+ at 45 °C under laboratory conditions [30]. However, the behavior and dependence on the temperature of the gene transcript levels and the enzymes involved in the genomic context of the main nitrogen assimilation are still unknown.

It was recently shown that stigonematal members, especially *Mastigocladus* spp., were the most active and relevant nitrogen producers at high temperatures in the Chilean Porcelana hot spring [2]. In that study, a representative of the Stigonematales denoted CHP1 (CHilean Porcelana 1) was isolated from the Porcelana microbial mat. Based on morphological features, such as type of branching, this strain was tentatively identified as belonging to the genus *Mastigocladus* [3]. Therefore, the aim of the present study was to corroborate the affiliation and study the genetic adaptation of the prominent and highly relevant phototrophic and diazotrophic thermophilic strain CHP1 with respect to the regulation of its nitrogen fixation and combined nitrogen assimilation at high temperatures. Strain CHP1 was evaluated morphologically (morphology and subcellular structures) and genetically (using ribosomal and functional marker genes) under controlled conditions by following growth, nitrogen fixation (nitrogenase enzyme activity), nitrogen assimilation (isotopic ^{15}N uptake) and the expression of related key genes (*nif*H, *nar*B, *gln*A) at various temperatures (45–60 °C). The data produced demonstrated the affiliation of strain CHP1 to the stigonematal thermophilic clade formed by *Mastigocladus* spp., and that this strain could perform optimally (growth and nitrogenase activity) at 45–50 °C. This metabolic temperature dependency of strain CHP1 was consistent with previous in situ results demonstrating the high nitrogen contribution of the stigonematal members in the Porcelana hot spring [2] from where this strain was isolated. Therefore, *Mastigocladus* sp. strain CHP1 is a suitable organism for studying thermophilic Stigonematales (subsection V) in order to answer certain questions concerning the cellular and genetic adaptations to high temperature used by phototrophic organisms inhabiting microbial mats.

Materials and methods

Strain CHP1 isolation and temperature growth curves

Strain CHP1 was collected at 46 °C from the Porcelana hot spring located in Northern Patagonia, Chile (42° 27′ 29.1″S–72° 27′39.3″W). An isolate (non-axenic) was obtained through the serial dilution technique under microscope observation until one unique filament was obtained. The filament was plated on sterile BG11 medium [49], and grown at three different temperatures, 45, 50 and 60 °C. Each plate was maintained in batch culture under a 12 h light–12 h dark photoperiod with 30 μmol photons m^{-2} s^{-1} of white light. After three weeks growth, the biomass formed was transferred to a borosilicate glass flask (250 mL) and maintained for one month under the conditions described above until sufficient biomass was obtained to perform the different temperature growth curves.

Growth curves were measured in batch cultures (500 mL flasks) using BG11 free-nitrogen ($BG11_0$) and BG11 medium supplemented separately with $NaNO_3$ (9 mM) ($BG11_{NO3}$) and NH_4Cl (200 μM) ($BG11_{NH4}$) at 45, 50 and 60 °C. Prior to starting the curves and any experiments, the CHP1 culture was washed using BG11-Triton and incubated with 5 mg mL^{-1} lysozyme overnight (ON) with stirring in the presence of kanamycin (100 μg mL^{-1}) and spectinomycin (20 μg mL^{-1}) in order to eliminate any accompanying bacteria. After this, the growth curves were initiated with an inoculum of 2 mg L^{-1} Chl *a* at each temperature and N supplement condition. The growth was measured as the increase in concentration of chlorophyll *a* (Chl *a*) content and dry weight (mg L^{-1}). Two replicates of 200 mL each were used for Chl *a* determinations, and six replicates of 200 mL each were used for dry weight measurements. Samples for Chl *a* determinations were obtained every three days and processed according to Chorus and Bartram [14] modified with an additional step in which the cells were homogenized by bead beating (4.0 ms^{-1} for 2 min) using solid glass beads (1 mm). Every 7 days over the course of 42 days, one replicate (200 mL flask) was filtered using Isopore™ membrane filters (10 μm pore size,

Merck Millipore), and the pellet was dried at 60 °C for determination of dry biomass.

DNA extraction for genome sequencing

The culture biomass of strain CHP1 grown at 50 °C was used for DNA extraction. For this, 250 mL of biomass was washed as described above. DNA extraction and purification were performed as described by Alcamán et al. [2]. The genomic DNA was then sequenced according to Rodrigue et al. [50]. The DNA molecules were end-repaired and phosphorylated using an end-repair kit (Enzymatics or New England Biolabs) according to the manufacturer's recommendations. The amplified libraries were subjected to an additional round of purification using AMPure XP SPRI beads to remove residual primers and adapter dimers. The samples were quantified by real-time PCR on a LightCycler II 480 (Roche). Illumina libraries were loaded on to an Illumina GAIIx sequencer, and the data were analyzed using the Illumina pipeline 1.4.0 to generate fastq files. The raw sequences were cleaned of barcode, and the quality was checked with FastQC software and filtering with Trimmomatic [7]. Finally, the CHP1 genome was partially assembled using the SPAdes-3.9.0 assembler [4], and annotated with PROKKA-1.11 software [56]. The CHP1 draft genome had a size of 5.9 Mb, 88 contigs, 4863 predicted proteins (CDS), one 16S rRNA and 40 tRNAs.

Only the genes of interest for the study (*nif*H, *nar*B, *nir*A, *gln*A and 16S rRNA) were then further analyzed and submitted to the NCBI database.

Phylogenetic analysis of Porcelana strain CHP1

RNAmmer software [35] was used to search the complete 16S rRNA gene sequence from strain CHP1 (the complete 16S rRNA gene was deposited in the public database under the accession number KX035101). Partial 16S rRNA gene sequences (~1300 bp) for all available Stigonematales and Nostocales from the SILVA SSU database were used for comparisons between subsections. All sequences were aligned using MEGA5.2 software [68] with the ClustalW algorithm (Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA). The substitution models used for the phylogenetic analysis were checked using the Model Selection (ML) option of MEGA 5.2 software based on the lowest BIC score obtained for each dataset [68]. The subsequent 16S rRNA gene phylogenetic reconstruction was performed using the maximum likelihood search strategy, Kimura's 2-parameter substitution model with G + I rates between sites, and 10,000 bootstrap replicates. Additionally, phylogenetic reconstruction of the *nif*H gene from available Stigonematales and Nostocales, including strain CHP1, was performed using the GTR substitution model and the procedure described above for the 16S rRNA gene sequences. The sequences of *Trichodesmium erythraeum* IMS101 and *Gloeobacter violaceus* PCC7421 were used as outgroups for the 16S rRNA gene, and *T. erythraeum* IMS101 was used as the outgroup for the *nif*H gene phylogenetic reconstructions.

Morphological characterization by light microscopy and TEM

Two independent 1 mL samples from each culture growth curve condition were fixed with 2.5% glutaraldehyde for morphological characterization. One sample was used for light microscopic morphological characterization using a Labophot-2 microscope (Nikon, Japan) coupled with a 100-W mercury lamp (LH-M100C-1 Model), and the other was used for transmission electron microscopy (TEM). The morphological characterization was performed according to Anagnostidis and Komárek [3], and the dimensions of vegetative cells in the branching and leader filaments were determined according to Kaštovský and Johansen [29]. For TEM, 500 μL of cells were fixed with 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) and maintained for 16 h at room temperature. The cells were washed with cacodylate buffer (0.1 M, pH 7.2) for 2 h at 4 °C, post-fixed with 1% (w/v) osmium tetroxide for 90 min, rinsed with distilled water, and stained with 1% uranyl acetate for 60 minutes. The cells were then dehydrated in acetone (50%, 70%, twice at 95%, and three times at 100%), polymerized in Epon:Acetone (1:1) overnight, and finally polymerized in Epon at 60 °C for 24 h. Ultra-thin sections (70 nm) were obtained on a Sorval MT-5000 ultramicrotome (MTS, USA), mounted on Formvar-coated copper grids and stained with 4% uranyl acetate followed by lead citrate. The samples were examined and photographed in a Philips Tecnai electron microscope (Philips, USA) operated at 80 kV with an integrated BioScan Megaview G2 ccd camera (Olympus-Sis, Japan). Heterocyst characterization was performed according to Nierzwicki-Bauer et al. [46].

Nitrogen assimilation assays in growing cultures

Based on the results of the growth culture curves, biomass in the exponential phase (previously washed as described above) was used to start the daily cycle of nitrogen fixation ($^{15}N_2$: ARA) and nitrogen assimilation ($K^{15}NO_3$ and $^{15}NH_4Cl$) assays. Nitrogen fixation was measured by the activity of the nitrogenase enzyme (acetylene reduction assays: ARA) and the isotopic technique ($^{15}N_2$ isotopic uptake). The ARA assay was performed at 45 and 50 °C, with three biological replicates (three culture flasks of 250 mL). Five milliliters of CHP1 culture from each replicate were placed in quadruplicate into sterilized 10 mL glass incubation vials. The assay began when 1 mL of air in each vial was replaced with 1 mL of acetylene gas (10–20% of the gas phase), and the vials were then incubated for 3 h at the indicated temperatures. In addition to the four replicates, two controls (one with CHP1 culture but no acetylene gas and one containing only acetylene gas) were included. The samples were incubated for six periods of 3 hours each (08:00–11:00; 11:00–14:00: 15:00–18:00, 19:00–22:00; 23:00–02:00; 03:00–06:00) over a diurnal cycle. From each vial, 4 mL of the gas phase were withdrawn using a hypodermic syringe and transferred to a 5 mL BD Vacutainer® (No Additive Z Plus tube, ref: 367624). The ethylene produced was analyzed by injecting 1 mL of the gas using a gas-tight syringe (Hamilton) into a GC-8A gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with an 80/100 Porapak™ Q 1 m × 1/4″ column (Supelco, St. Louis, MO, USA) and a flame ionization detector with helium as the carrier gas. The nitrogenase activity was calculated from the ethylene produced, which was corrected based on the controls and expressed per dry weight and incubation time.

In parallel to the ARA assays, $^{15}N_2$ uptake experiments were performed in 150 mL polycarbonate bottles. The ^{15}N assimilation experiments (N_2) were initiated through the addition of 1 mL of $^{15}N_2$ gas (98 atom % $^{15}N_2$ gas, Sigma-Aldrich) to the bottles through a gas-tight syringe. In addition, two replicates of 20 mL each (without isotope $^{15}N_2$) at each temperature (45 and 50 °C) were collected to determine the natural isotopic composition (control). Six bottles were inoculated at the same time (08:00 h) and one bottle was harvested after 3, 6, 10, 14, 18 and 24 h. After each incubation period, the content of each bottle was filtered through an Isopore™ membrane filter (10 μm pore size, Merck Millipore), and the pellets obtained were dried at 70 °C for 48 h. Measurement of ^{15}N atom incorporation (AT ^{15}N) and particulate organic nitrogen (PON) was performed using a mass spectrometer (IRMS Delta V Advantage, Thermo Finnigan, Laboratory of Biogeochemistry and Applied Stable Isotopes: LABASI). Calculation of the ^{15}N rate was performed as described by Alcamán et al. [2] including corrections for dilution of $^{15}N_2$ gas and controls.

Isotopic ammonium ($^{15}NH_4$) and nitrate ($^{15}NO_3$) assimilation experiments using isotopic solutions ($^{15}NH_4Cl$ and $K^{15}NO_3$, respectively) were performed independently on cultures previously grown at 45 °C in $BG11_{NO3}$ and $BG11_{NH4}$. The experiments began by washing the cultures with distilled water in order to eliminate debris and nitrogen sources. Each culture was then resuspended in $BG11_0$ and maintained in darkness for six hours to synchronize the circadian rhythms of the cyanobacteria [31]. The washed pellet from $BG11_{NO3}$ was divided into two portions; one of which was inoculated into BG11 medium containing KNO_3 (9 mM) and $K^{15}NO_3$ (1 mM) in triplicate, and the other was inoculated into BG11 $^{15}NH_4Cl$ medium (200 μM) in triplicate. The same procedure was carried out for the washed pellet from $BG11_{NH4}$. From each assay, six samples were collected in triplicate over a 48 h cycle. Subsamples for RNA, nutrients (NO_3^- and NH_4^+) and isotopic analysis were also obtained at 3, 6, 12, 18, 24 and 48 h. Nutrient analysis was performed using an autoanalyzer (Seal Analytical AA3; Biogeochemistry Laboratory, Universidad of Concepción, Chile), and isotopic analyses were carried out as described above.

Nitrogen assimilation related genes in strain CHP1

Reference sequences from *Anabaena* sp. PCC7120, *Fischerella* sp. NIES 3754 and other cyanobacteria [23,25,28,54] were used to search for the *gln*A, *nar*B, *nif*H and *nir*A genes in the genome of strain CHP1, and they were assigned accession numbers: KX035102, KX035103, KX035104 and KX035105, respectively. Moreover, data obtained from the US DOE JGI IMG (https://img.jgi.doe.gov/cgi-bin/m/main.cgi) and PATRIC (https://www.patricbrc.org/portal/portal/patric/Home) databases for *Fischerella* sp. PCC 9431 (ID 2512875027), *M. laminosus* UU774 (ID 1594576), *Fischerella* sp. JSC-11 (ID 2505679024) and *Fischerella* sp. NIES-3754 (ID 1752063), which are representative of mesophilic and thermophilic cyanobacteria, were used to compare the synteny of nitrogen metabolism-related genes. For all genomes, once the region in the genome was located, the sequences in the surrounding regions were extracted and examined in the ORF Finder program (http://www.bioinformatics.org/sms2/orf_find.html) in order to define the open reading frames (ORFs) in the DNA sequence. This program returns the range of each ORF, along with its protein translation. Each ORF found was then blasted in the NCBI database using the BLASTP and BLASTN tools in order to assign potential protein encoding segments. This search allowed the identification of neighboring genes, and therefore the genomic cluster context of each gene.

RNA extraction from N assimilation experiments and RT-qPCR for gene expression analysis

Three biological replicates from the isotopic assays were used for analysis of gene expression by RNA analysis. These samples were collected from the cells grown in $K^{15}NO_3$ and $^{15}NH_4Cl$ at 45 °C (at 3, 6, 12 and 24 h), and samples were also collected for nitrogen fixation at 4, 8, 12, 16, 20 and 24 h at the two temperatures investigated (45 and 50 °C). RNA from the samples was extracted using Trizol and the RNA Clean & Concentrator™ kit (Zymo Research, USA). The quality and quantity of the RNA were determined using a spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and by electrophoresis in an RNase-free 1% agarose gel. DNase treatment (TURBO, Applied Biosystems, USA) was performed, and 1.5 μg of RNA from each sample (in triplicate) was used for qPCR standardization. Then, cDNA was synthesized using the ImProm-II™ Reverse Transcription System (Promega, USA) according to the manufacturer's specifications. For qPCR measurement of *nif*H, *nar*B and *gln*A gene expression, specific primers for strain CHP1 were designed (Supplementary data, Table S1). Each PCR product obtained was cloned into the pGEM-T® (Promega, USA) vector plasmid, and a plasmid stock (10^{10} copies) and plasmid curve (10^2–10^8 copies) were obtained. The SensiMix kit (Bioline, USA) was used to detect the fluorescence signals. Real-time qPCR (Roche LC 480) was run as follows: 40 cycles at 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 30 s. All primers were designed to have a similar annealing temperature in order to run under similar conditions. To normalize the quantification of transcripts, three different housekeeping genes were tested. Stability analyses for *pet*B, 16S rRNA and *ilv*D gene transcripts in the different media ($BG11_0$, $BG11_{NO3}$ and BG_{NH4}) were conducted [48]. According to the geNorm algorithm, the most stable gene under the tested conditions was the *ilv*D housekeeping (HK) gene (Supplementary data, Fig. S1). All gene quantifications were extrapolated using the respective plasmid standard curve, and normalized based on the absolute quantification of the *ilv*D HK gene.

Results

Phylogenetic affiliation of Porcelana strain CHP1

A phylogenetic reconstruction was performed first based on the complete 16S rRNA and *nif*H gene sequences in all sequenced genomes of *Stigonematales* cyanobacteria (subsection V) and on some other selected sequences from the SILVA SSU database. Reconstruction using the 16S rRNA gene (Fig. 1) demonstrated that Porcelana strain CHP1 clustered (bootstrap 97%) exclusively with members of the genera *Mastigocladus* and *Fischerella*. All members within this clade were characterized as thermophilic, whereas the remaining sequences obtained from stigonematal members were non-thermal and clustered separately (*Fischerella*, *Hapalosiphon*, *Westiellopsis*, *Nostochopsis*, *Westiella* and *Mastigocladus*; bootstrap 95%) from the thermal clade. Since all the clade members were diazotrophs, a second phylogenetic reconstruction based on the complete *nif*H gene was performed (Supplementary data; Fig. S2). This reconstruction confirmed the affiliation of strain CHP1 within the thermophilic Stigonematales and showed that it formed a tight clade (99% sequence identity) with *Mastigocladus* and *Fischerella* spp. isolated from hot springs.

To widen the affiliation basis further, strain CHP1 was examined morphologically under light (LM; Fig. 2) and transmission electron microscopy (TEM; Figs. 3 and 4). LM examination showed that the principal filaments were mostly uniseriated, with true lateral branches due to cell division in more than one plane. The vegetative cells were on average 7.6 ± 1.3 (SD) μm long and 6.0 ± 1.8 μm wide, whereas branched cells were 8.0 ± 2.1 μm long and 4.8 ± 1.4 μm wide (Fig. 2). As seen in Fig. 2, branching of the T-Bd (T-bd) and Y-Di (Y-di) types was observed (Fig. 2b and e), and both V- and X-branching types were occasionally present (Fig. 2b).

After growth on different nitrogen sources (atmospheric nitrogen gas-$BG11_0$ and the combined nitrogen sources nitrate-$BG11_{NO3}$ and ammonium-$BG11_{NH4}$), all vegetative cells were characterized by strong red chlorophyll *a* fluorescence (Fig. 2g–i), in contrast to the non-fluorescent heterocysts that developed in the absence of a combined nitrogen source (Fig. 2g). The heterocyst/vegetative cell frequency was 2–4% irrespective of temperature. Heterocysts in primary trichomes were predominantly intermediate in location and less frequently terminal (Fig. 2a, d, and g). Ultrastructural (TEM) analyses of strain CHP1 revealed no major differences between cells grown at the various temperatures or under the different nitrogen regimes examined (Fig. 3). Both proheterocysts and mature heterocysts showed the subcellular reorganization expected for vegetative cells, including the development of extra wall layers, a

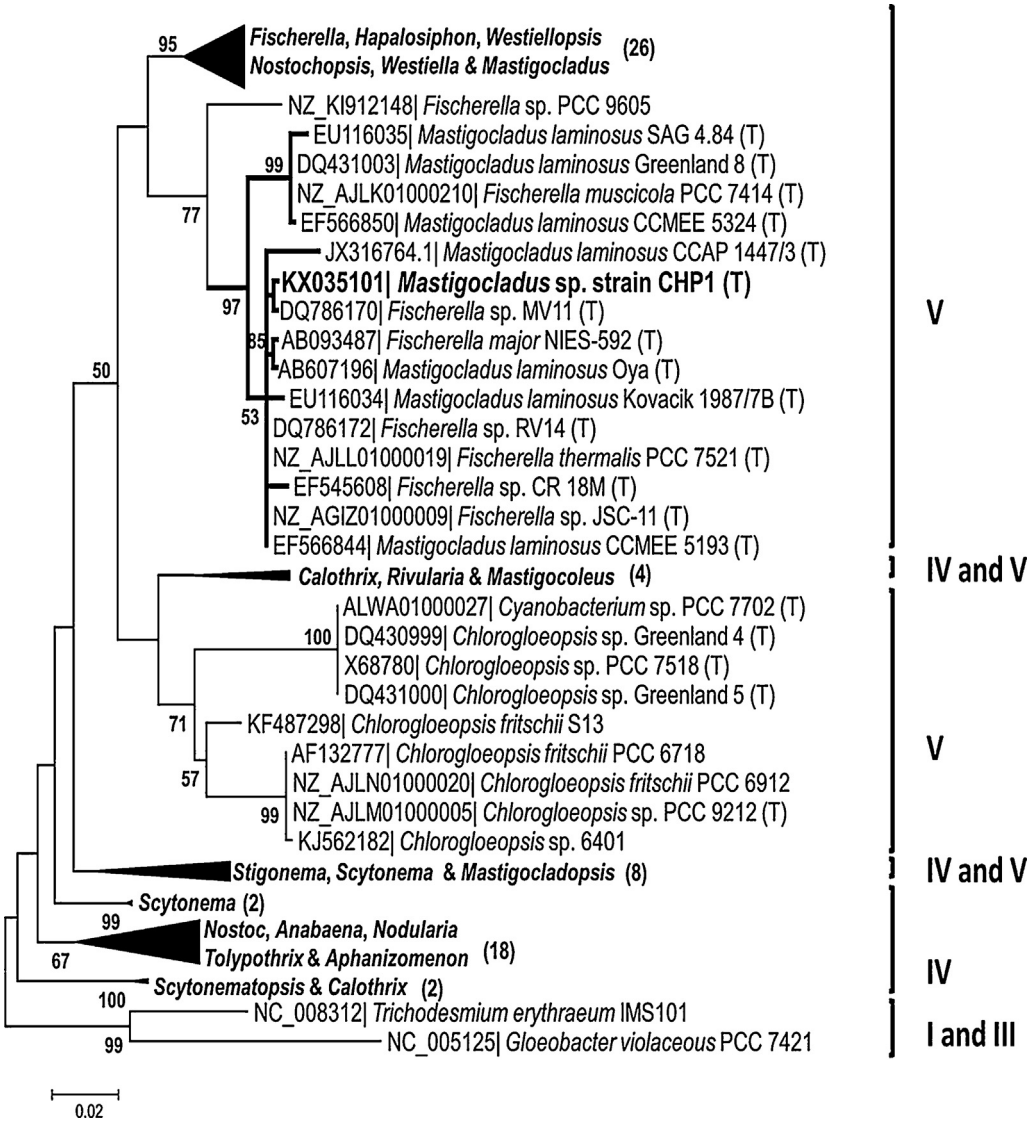

Fig. 1. Phylogenetic reconstruction based on the 16S rRNA genes of major representatives of the filamentous cyanobacteria. The affiliation of sequences classified as *Mastigocladus* sp. strain CHP1 is clearly located within the order Stigonematales (heterocystous branching Subsection V). The thick black lines show clades whose representatives are most closely related to *Mastigocladus* sp. strain CHP1. All strains with thermal origin were labelled with (T), stressing the close phylogenetic relatedness among the thermophiles. The cyanobacterium *Trichodesmium erythraeum* IMS101 (subsection III) and the unicellular *Gloeobacter violaceus* PCC7421 (subsection I) were used as outgroups. The numbers at the nodes represent bootstrap values of 10,000 replicates.

narrow neck leading to adjacent vegetative cells, and an extensive reorganization of the thylakoid membranes (Fig. 4).

Since all these features, specifically the uniseriate filaments, true lateral branching and a vegetative cell size of 8–10 μm, are typical of members of the genus *Mastigocladus*, it was concluded that strain CHP1 affiliated with *Mastigocladus* sp.

Thermotolerance under different nitrogen regimes

To test the genetic adaptation of strain CHP1 based on its thermophilic characteristics and nitrogen requirements, growth at various temperatures in the presence of different nitrogen sources was measured. Growth in $BG11_0$ was measured at 45, 50 and 60 °C and growth in $BG11_{NO3}$ and $BG11_{NH4}$ medium was measured at 45 and 50 °C. Exposed culture in $BG11_0$, $BG11_{NO3}$ or $BG11_{NH4}$ (Fig. 5a) at 60 °C only showed initial growth, but after 3 days under this condition the culture started showing a chlorotic appearance but after 6 days the culture looked dead (data not shown). In contrast, at lower temperatures (45 and 50 °C) the biomass (dry weight) of strain CHP1 increased substantially over time under both the $BG11_0$ and $BG11_{NO3}$ regimes (Fig. 5a and b). The biomass in $BG11_0$, which was initially low, was followed by a more significant increase, reaching 66.1 mg after 38 days at 50 °C. However, biomass yield was consistently highest under $BG11_{NO3}$ (9 mM $NaNO_3$), reaching 166.3 mg at 45 °C and 122.8 mg at 50 °C. The increase in biomass measured as chlorophyll *a* showed a similar pattern (Fig. 5a and b).

In previous studies it was suggested that optimal ammonium concentrations for cyanobacterial growth were in the range of 2–10 mM NH_4Cl. However, 2 mM NH_4Cl caused severe bleaching in strain CHP1 cells after day 6 (data not shown) and, therefore, concentrations of 0.02, 20 and 200 μM ammonium in $BG11_{NH4}$ medium were tested. NH_4Cl at 0.02 μM (closest concentration found in the Porcelana hot spring [2]), showed the heterocyst appearance in the filaments of strain CHP1 around day 4. However, the CHP1 culture exposed at 20 μM and 200 μM NH_4Cl, developed heterocysts after 6 and 21 days, respectively. According to this, the concentration of 200 μM NH_4Cl was selected for comparative examinations (Fig. 5c). At both temperatures, growth on NH_4Cl

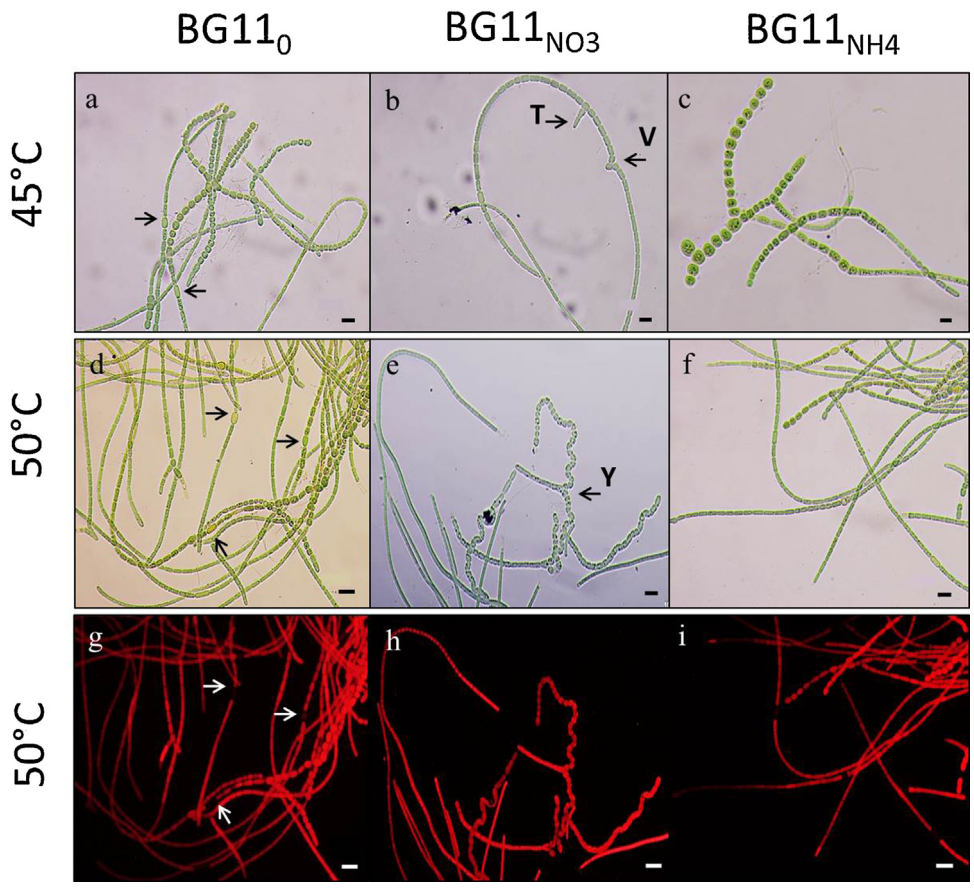

Fig. 2. Morphological appearance of *Mastigocladus* sp. strain CHP1 in light and epifluorescence microscopy grown under different nitrogen sources and temperatures. (a and d) Growth under nitrogen-fixing conditions (BG11$_0$). Heterocysts are apparent as more transparent and larger cells that are mostly intercalary (black arrows). (b and e) Growth under BG11$_{NO3}$ and (c and f) BG11$_{NH4}$ conditions. Non-heterocystous uniseriate filaments and filaments with type T, V and Y branching points predominated under all nitrogen regimes. (g–i) Epifluorescence micrographs showing the red fluorescence of chlorophyll in vegetative cells under all growth conditions. (g) The lack of epifluorescence in heterocysts is indicated (white arrows). Bar represents 10 μm.

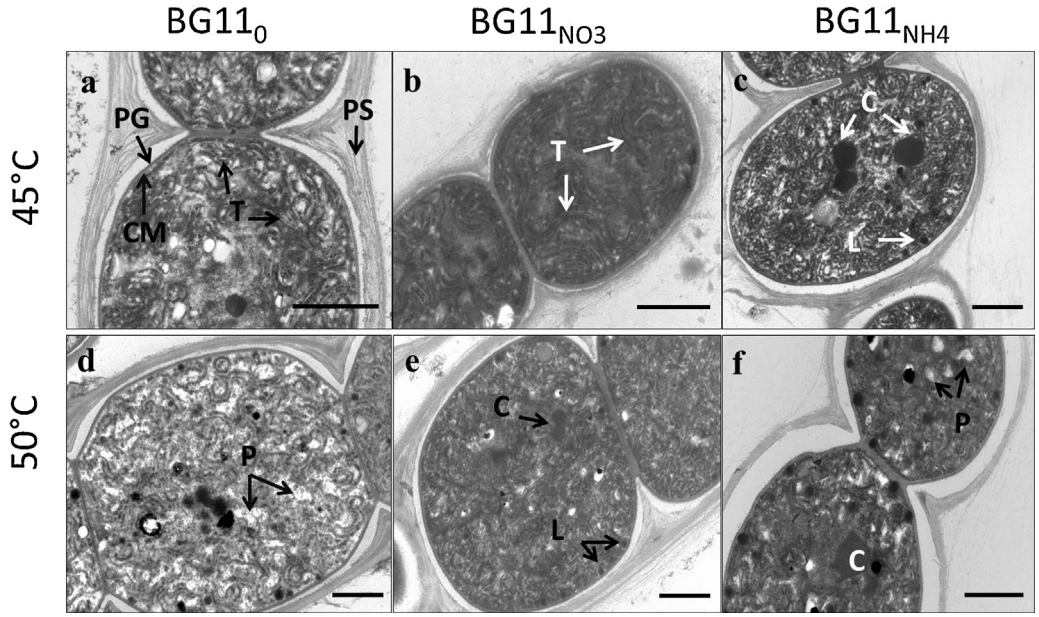

Fig. 3. Electron micrographs of ultrathin sections of vegetative cells of *Mastigocladus* sp. strain CHP1 grown under different nitrogen sources and temperatures. (a and d) Cells grown under nitrogen-fixing conditions (BG11$_0$) (i.e. in the absence of a combined nitrogen source). (b and e) Cells grown on BG11$_{NO3}$ and (c and f) on BG11$_{NH4}$ at the indicated temperatures. Abbreviations: PS, external polysaccharide layers; PG, peptidoglycan layer; CM, cytoplasmic membrane; T, thylakoids; C, carboxysomes; L, lipid bodies; P, polyphosphate granules. The bar corresponds to 1 μm.

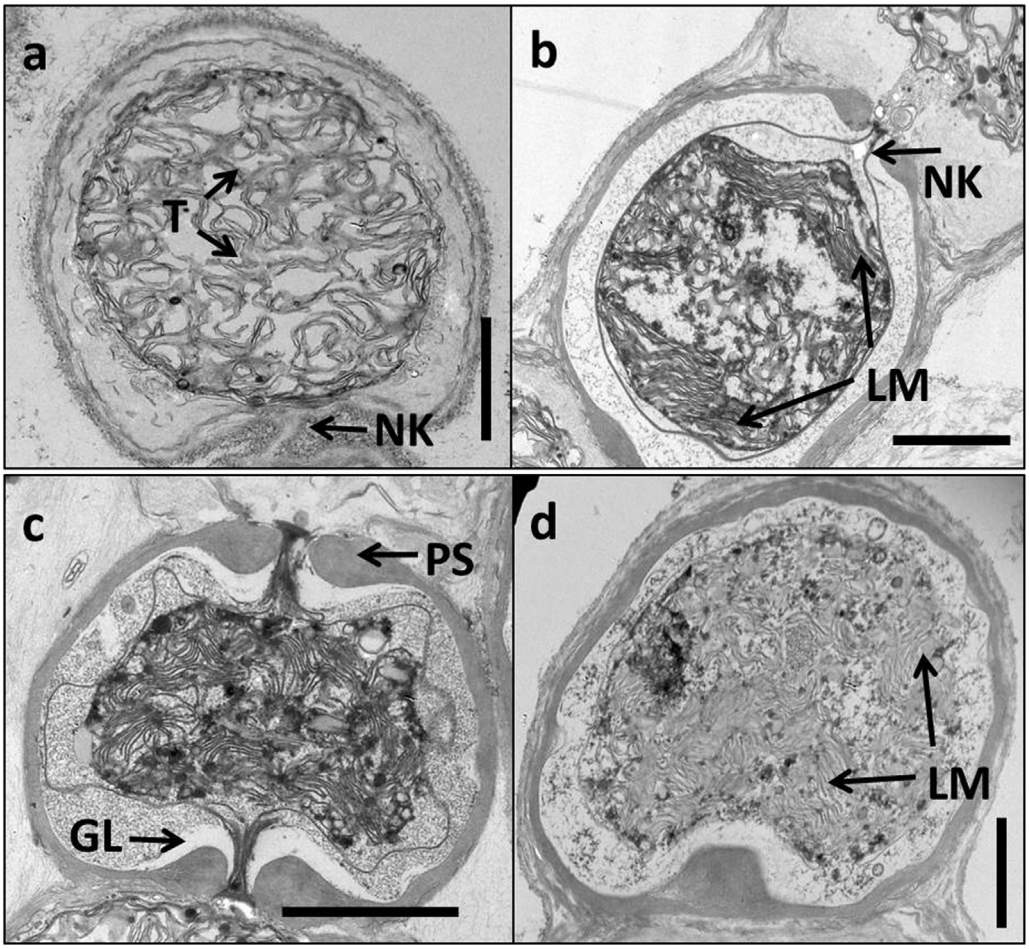

Fig. 4. Ultrastructural characteristics of developmental stages of heterocysts of *Mastigocladus* sp. strain CHP1 grown under nitrogen-fixing conditions ($BG11_0$). (a) Proheterocyst at an early stage of development characterized by loose external cell wall layers, rearrangement of thylakoids (T), and narrowing of the neck (NK) leading to the adjacent vegetative cell. (b) Intermediate developmental stage of the heterocyst, characterized by a narrow neck (NK), external cell wall layers that are still not fully developed and lamellar membranes (LM). (c and d) Mature heterocysts with distinct bundles of stacked, lamellar membranes (LM) filling up the entire cytoplasm, well-defined narrow necks, external polysaccharide layers (PS) and glycolipid layers (GL). Note the absence of other subcellular structures of vegetative cells at all stages (compare with Fig. 3). Bar, 2 μm.

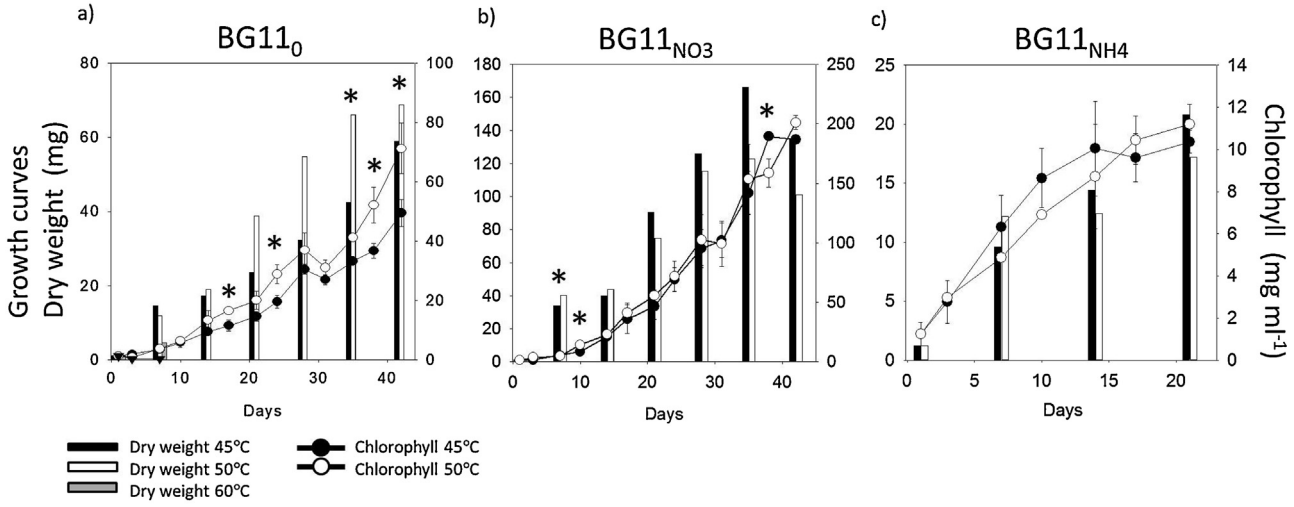

Fig. 5. Growth of *Mastigocladus* sp. strain CHP1 under different nitrogen regimes and temperatures. (a) Under nitrogen-fixing conditions (i.e. in the absence of a combined nitrogen source): $BG11_0$; (b) in a medium supplemented with KNO_3 (9 mM): $BG11_{NO3}$; and (c) in medium supplemented with NH_4Cl (200 μM): $BG11_{NH4}$. Each culture initially contained 2 mg chlorophyll L^{-1}. The cultures in $BG11_0$ were incubated at 45, 50 and 60 °C, and the cultures in $BG11_{NO3}$ and $BG11_{NH4}$ were incubated at 45 and 50 °C. Biomass is given as dry weight (mg) and chlorophyll *a* (mg mL^{-1}) during a growth period of six weeks, except in (c). Note differences in the scales of the Y-axes. Growth in $BG11_0$ at 60 °C ceased after 6 days (gray bars). Asterisks denote significant differences ($p < 0.05$) by the Student's t-test for chlorophyll *a* at the two temperatures.

measured as dry weight or chlorophyll *a* was considerably lower than growth in medium containing NO_3, reaching 20.8 mg at 45 °C and 17.2 mg at 50 °C, approximately 20-fold lower than when the cultures were grown in $BG11_{NO3}$ (Fig. 5b).

Taken together, these results showed that strain CHP1 was clearly thermotolerant up to approximately 50 °C, and that its thermotolerance was influenced by the prevailing nitrogen regime. Nitrogen fixation ($BG11_0$) consistently supported the highest growth at 50 °C, whereas nitrate ($BG11_{NO3}$) supported the highest growth at 45 °C, and under the latter conditions growth was 2–3 times higher. Growth under $BG11_{NH4}$ showed no clear thermopreference and was many-fold lower than under the other nitrogen regimes.

Nitrogen assimilation capacity and rate

The nitrogen assimilation capacity of the CHP1 culture was examined next. Nitrogen fixation rates during light–dark regimes were examined both by $^{15}N_2$ assimilation assay and by acetylene reduction assay (nitrogenase activity). As seen in Fig. 6, exposing cultures to $^{15}N_2$ gas at 45 and 50 °C resulted in $^{15}N_2$ gas uptake of 7.5 (at 50 °C) and 8.3 (at 45 °C) nmol PON mg^{-1} throughout the light/dark cycle. The activity was 2-fold higher at midday and showed a marginal effect of temperature (Fig. 6a). Nitrogenase activity (acetylene to ethylene) was recorded throughout the light/dark period, whereas for the isotopic uptake, there was a distinct peak at midday, reaching 54.3 ± 3.9 (SD) nmol ethylene mg^{-1} produced at 45 °C, and up to 62.4 ± 10.8 (SD) nmol ethylene mg^{-1} at 50 °C.

At 45 °C, the cellular uptake of nitrate as $K^{15}NO_3$ in cultures previously grown in $BG11_{NO3}$ was light-dependent, ranging from 342 ± 14.7 (SD) to 412 ± 9.5 (SD) nmol N mg^{-1} h^{-1}. In cells pregrown in $BG11_{NH4}$, the uptake was up to 10-fold lower (28 ± 6.2 (SD) to 54 ± 2.1 (SD) nmol N mg^{-1} h^{-1}; Fig. 6b). The concentration of nitrate in the $BG11_{NO3}$ medium remained high during the 48-h time course of the experiment.

Likewise, cellular uptake of ammonium as $^{15}NH_4Cl$ (Fig. 6c) was higher in cultures previously grown in $BG11_{NH4}$ than in cultures previously grown in $BG11_{NO3}$, although this uptake was considerably lower than the $K^{15}NO_3$ uptake (Fig. 6b). A maximum incorporation of 48 ± 3.7 (SD) nmol N mg^{-1} h^{-1} was found after 18 h. Ammonium levels in the medium approached zero after 48-h incubation (Fig. 6c), whereas high nitrate levels still remained in the medium after this period (Fig. 6b).

Expression of key genes in the nitrogen fixation and assimilation pathways

Fluctuations in the transcript levels of *nif*H (encoding the Fe subunit of the nitrogenase complex), *gln*A (encoding the ammonium-assimilating enzyme, glutamine synthetase) and *nar*B (encoding the nitrate reductase involved in nitrate assimilation) genes in strain CHP1 were followed during light/dark cycles using RT-qPCR, combined with normalization to the expression of the *ilv*D HK gene [48]. The *nif*H gene in strain CHP1 was only expressed in the presence of mature heterocysts (which occurred in 2–4% of the total number of CHP1 cells growing in $BG11_0$ medium). At 45 °C, *nif*H gene transcript levels increased under the dark condition, while at 50 °C the *nif*H gene transcript levels were higher under the light condition and decreased markedly (from 1500 to 100 *nif*H transcripts) during the dark condition (Fig. 7a). For the *gln*A gene, a high expression was expected, as it is typically reported to be expressed in nitrogen-fixing heterocysts as well as in all vegetative cells. Transcription of the *gln*A gene (normalized to the *ilv*D HK gene) in strain CHP1 cells under nitrogen-fixing conditions (growing in $BG11_0$ medium) was high at 50 °C under the light condition (7928 gene copies), as seen in Fig. 7b, although pronounced *gln*A activity was also recorded at both temperatures in the dark period (5800 and 3195 transcripts at 50 and 45 °C, respectively).

In order to understand the transcription performance of strain CHP1 after pre-adaptation of nitrogen sources (ammonium and nitrate), the transcript levels of *nif*H, *gln*A and *nar*B genes were measured by RT-qPCR in 24 h experiments at a temperature of 45 °C (Fig. 8). Strain CHP1 pre-adapted to nitrate and supplemented with $^{15}NO_3$ ($BG11_{NO3/15NO3}$), showed high *gln*A and *nar*B gene expression in light conditions (day time), reaching 1853 ± 263 (SD) and 1645 ± 150 (SD) transcripts, respectively (Fig. 8a). These expression levels were one order of magnitude higher than those observed for strain CHP1 pre-adapted to ammonium and supplemented with $^{15}NO_3$ ($BG11_{NH4/15NO3}$) (*gln*A 443 ± 110 (SD) and *nar*B 361 ± 17 (SD) transcript genes) (Fig. 8b). A similar pattern was observed in cultures supplemented with $^{15}NH_4Cl$ ($BG11_{NO3/15NH4}$ and $BG11_{NH4/15NH4}$) (Fig. 8c and d). In this case, under $BG11_{NO3/15NH4}$, the *gln*A and *nar*B genes reached 636 ± 75 (SD) and 490 ± 97 (SD) transcripts, respectively (Fig. 8c), whereas under $BG11_{NH4/15NH4}$ conditions, the *gln*A gene reached 870 ± 498 (SD) transcripts and the *nar*B gene 344 ± 78 (SD) transcripts (Fig. 8d). According to this, strain CHP1 pre-adapted with nitrate as a nitrogen source showed higher transcription levels of both *gln*A and *nar*B genes compared to ammonia pre-adaptation (Fig. 8b–d).

Nitrogen cluster gene organization

Next, the genic context of the *nif*HDK gene cluster in the genome of strain CHP1 was investigated and compared with that of *M. laminosus* UU774, *Fischerella* sp. JSC-11, *Fischerella* sp. NIES-3754 and, finally, with *Fischerella* sp. PCC9431, a mesophilic representative of the Stigonematales. The *nif*HDK cluster of strain CHP1 had synteny with the gene clusters recovered from the reference genomes used for comparison (Fig. S3), except for *M. laminosus* UU774 and *Fischerella* sp. PCC9431, which, in the latter, was interrupted by genes encoding hypothetical proteins, a phage integrase and a methylase protein. In addition, extra copies of *nif*D and *nif*K were also found near the *nif*HDK cluster in strain CHP1. The *nar*B and *nir*A genes, which are related to nitrate and nitrite assimilation, respectively, formed a complete *nir*A-*nrt*ABCD cluster in the genome of strain CHP1 (Fig. S4), but were located on the complementary strand. Although the *nar*B gene was located in another region of the CHP1 genome, it was in synteny with the *nar*B gene of *M. laminosus* UU774 and *Fischerella* sp. JSC-11. In contrast to most other freshwater strains of cyanobacteria, the bi-specific (nitrate and nitrite) *nrt*P transporter was not detected in the genome of strain CHP1. Finally, the *gln*A gene in CHP1 showed no synteny with the *gln*A context of adjacent genes in the other genomes analyzed (Fig. S5).

Discussion

Filamentous cyanobacteria isolated from various thermal ecosystems worldwide have often been characterized as members of subsection V of the order Stigonematales [41], and particularly as *M. laminosus* Cohn ex Kirchner 1898, in accordance with the morphotype classified as *M. laminosus* [3,29].

From our phylogenetic (16S rRNA and *nif*H genes) and morphological analyses, it was apparent that strain CHP1 isolated from the neutral pH Porcelana hot spring in Chile was a member of the order Stigonematales (subsection V), family *Mastigocladaceae* Geitl. 1925, subfamily *Mastigocladoideae* and genus *Mastigocladus* Cohn 1862, to which a >98–99% 16S rRNA genetic identity was found. Furthermore, the morphological features identified in strain CHP1

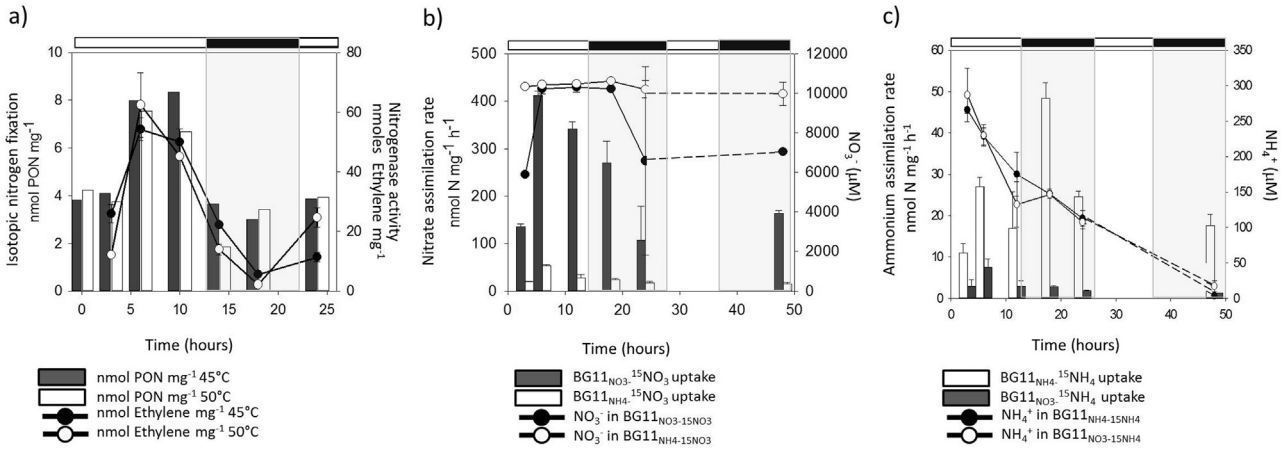

Fig. 6. Nitrogen assimilation in *Mastigocladus* sp. strain CHP1 during light/dark cycles at different temperatures. (a) Assimilation under nitrogen-fixing conditions using: (i) $^{15}N_2$ incorporation, given as particulate organic nitrogen (PON) formed (gray and white vertical bars), and (ii) acetylene reduction (ARA; nitrogenase activity), reported as the amount of ethylene produced (black and white circles). (b) Nitrate assimilation: growth in $BG11_{NO3}$ (gray bars) and $BG11_{NH4}$ (white bars) was followed by growth on isotopic $K^{15}NO_3$ added at time zero. The black and white circles show the nitrate concentrations during the experiment. (c) Ammonium assimilation: growth in $BG11_{NH4}$ (gray bars) and $BG11_{NO3}$ (white bars) was followed by growth on $^{15}NH_4Cl$ at time zero. The black and white circles show the ammonium concentrations during the experiment. Note the different scales of the Y-axes. The horizontal bars above the graphs indicate the duration and pattern of the light/dark cycles during the 24- or 48-h cycles.

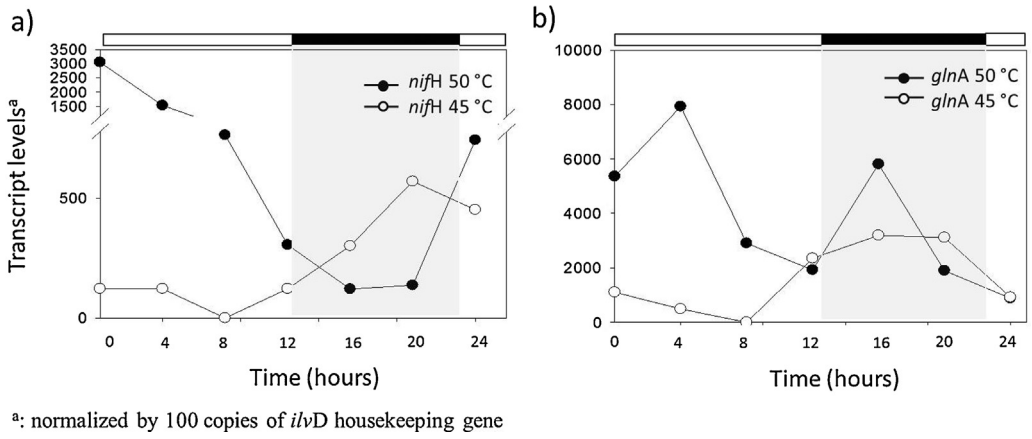

Fig. 7. Transcript levels of genes involved in atmospheric nitrogen fixation in *Mastigocladus* sp. strain CHP1. Transcript levels of (a) the *nif*H and (b) the *gln*A genes at 45 and 50 °C under nitrogen-fixing conditions ($BG11_0$) during light–dark transitions. The horizontal bars above the graph indicate the duration of the light and dark periods of the experiment.

(e.g. uniseriate leading strand, true branching of types T and Y), including its subcellular organization (e.g. a central distribution of thylakoid membranes), suggested a taxonomic affiliation specifically with *M. laminosus* [22,55,45,3]. The fact that this strain was unable to survive at 60 °C under any of the nitrogen regimes tested, but could grow rapidly at 45 and 50 °C classified the *Mastigocladus* sp. strain CHP1 as a moderately thermophilic cyanobacterium. In general, incubation at 50 °C promoted more rapid growth than incubation at 45 °C, which may reflect a potential genetic adaptation of the strain to 50 °C.

Similar thermotolerance with greater fitness at 40–55 °C was exhibited for other *Mastigocladus* spp. isolated from Yellowstone National Park (YNP) [43]. Hence, the previous tentative affiliation of strain CHP1 to *Mastigocladus* [2] has now been confirmed, as is the fact that this strain belongs to a clade exclusive for thermophilic or thermotolerant representatives of the genera *Mastigocladus* and *Fischerella*. The latter features further stress the close relationship between thermotolerant members of this clade, irrespective of their genus affiliation or geographic origin, as noted by Kaštovský and Johansen [29].

Our data also demonstrate that *Mastigocladus* sp. strain CHP1 had the capacity to grow under different nitrogen regimes, as well as on atmospheric dinitrogen gas as if under natural conditions in the Porcelana hot spring [2], although at dissimilar rates. In addition to the appreciable growth sustained on nitrogen gas ($BG11_0$), the most prolific growth of *Mastigocladus* sp. strain CHP1 occurred in the presence of nitrate ($BG11_{NO3}$) at both 45 and 50 °C, whereas growth was minimal in a medium supplemented with ammonium. These observations are consistent with data obtained for a thermophilic *M. laminosus* strain from Jakrem hot spring in Meghalaya, India [30] but not with data obtained for other *Mastigocladus* populations, such as those from White Creek and Boiling River in YNP, since the latter two populations showed similar growth under $BG11_0$ and $BG11_{NO3}$ regimes [42]. This might illustrate differences in nitrogen assimilation preferences with specific physiological adaptations between thermotolerant strains belonging to this subsection V. These differences suggest that physiological diversifications and adaptations of strains have occurred within the thermal *M. laminosus* clade due to the various physical and geochemical conditions to which members of the clade have been exposed [41].

As expected, nitrogen fixation in the heterocystous *Mastigocladus* sp. strain CHP1 grown under controlled conditions peaked in light irrespective of temperature (45 and 50 °C), and was abolished

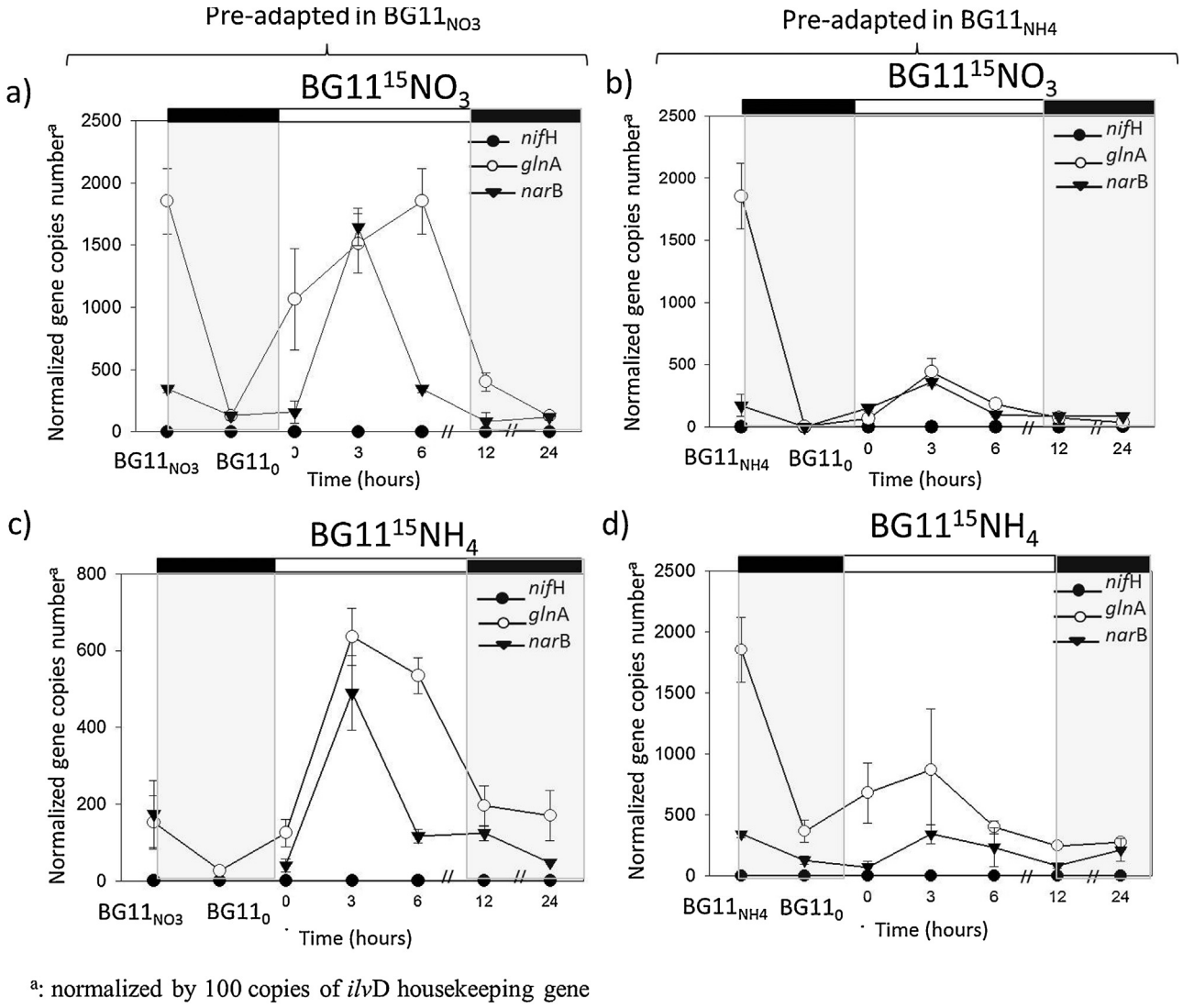

Fig. 8. Transcript levels of genes involved in pre-adapted nitrogen assimilation in *Mastigocladus* sp. strain CHP1 at 45 °C. Transcript patterns of *nif*H, *gln*A and *nar*B genes in cultures pre-adapted by growth in $BG11_{NO3}$ (a and b) and $BG11_{NH4}$ (c and d) prior to the start of the experiment. At time 0, the cultures were inoculated with (a and c) $K^{15}NO_3$ and (b and d) $^{15}NH_4Cl$. The gray area represents the dark periods, the first of which is a 6-h synchronization period in darkness prior to the start of the 24-h cycle. The horizontal bars above the graph indicate the durations of the light and dark periods of the experiment.

under combined nitrogen regimes. Maximum activity coincided with that found in situ at Porcelana hot spring [2], as well as with that of another thermophilic *M. laminosus* isolate from YNP [42]. Additionally, *nif*H gene expression in *Mastigocladus* sp. strain CHP1 was higher under light conditions at 50 °C, which was similar to previous observations found in situ in Porcelana at a temperature range of 48–58 °C [2]. Even though nitrogenase activity contribution by Stigonematales was associated with the presence and transcript levels obtained for the *nif*H gene [2], strain CHP1 was not the only stigonematal in the Porcelana hot spring mat and other populations also belonging to this genus could have been responsible for the activity recorded at temperatures up to 58 °C. On the other hand, *nif*H transcript levels were generally low and fluctuated moderately during light/dark cycles at 45 °C. There is also the possibility that *nif*H gene transcription may not be affected by circadian control at 45 °C. This is supported by the finding that *nif*H gene expression was constant under constant low light (30 μmol m^{-2} s^{-1}) in *Mastigocladus* sp. strain CHP1 (45–50 °C) (Arancibia-Loewe et al., unpublished). Similar behavior has been observed in the heterocystous cyanobacterium *Anabaena* sp. PCC 7120 (subsection IV), in other words there is a lack of rhythmicity in *nif*HDK gene expression under low light (30 μmol m^{-2} s^{-1}). The latter transcription profile is therefore classified as arrhythmic under low-light conditions [33]. Consequently, a deeper understanding of the overall molecular regulation of nitrogen fixation in *Mastigocladus* sp. strain CHP1 grown under different temperature and light regimes is now warranted.

The organization of the *nif*HDK gene cluster in the genome of *Mastigocladus* sp. strain CHP1 had close synteny with that of *Fischerella* sp. JSC-11 (Supplementary data, Fig. S3). Synteny of the *nif*HDK cluster between stigonematal cyanobacteria, such as *Fischerella* sp. and *M. laminosus*, has previously been demonstrated [54,60], suggesting that the *nif*HDK gene cluster closely resembles that of the *Mastigocladus* sp. strain based on the information available for the CHP1 genome.

Under nitrogen-fixing conditions at 50 °C, transcription of the *gln*A gene, which forms the basis of primary ammonia assimilation in all cell types, was high in *Mastigocladus* sp. strain CHP1, and a positive correlation between *nif*H and *gln*A gene expression patterns was observed. The presence of two distinct *gln*A transcription peaks in the light and dark is well-known for cyanobacteria

(e.g. the heterocystous *Anabaena* sp. PCC7120 [33] and the unicellular *Crocosphaera watsonii* WH8501 [58]). Moreover, the high transcript levels of the *nar*B gene, which encodes ferredoxin-dependent nitrate reductase found in light and nitrate-amended ($BG11_{NO3/15NO3}$) cultures, contrasted with the low transcript levels found during preadaptation in ammonium, suggesting severe inhibition of the *nrt*ABCD transporter under ammonium growth conditions [20,1]. The low ammonium tolerance ($\leq$200 μM) found in *Mastigocladus* sp. strain CHP1 may potentially trigger the minor changes observed in *gln*A transcription. A rapid decrease in glutamine synthetase activity on ammonium upshift has been shown for unicellular *Synechocystis* sp. PCC6803 [40]. Differences in NH_4^+ tolerance among cyanobacteria are well-known [16], may occur due to pH shifts [65], and may trigger disruption of the manganese cluster in the photosystem II oxygen-evolving complex [5,18].

On the other hand, our data show similar efficiencies for ammonium and nitrate uptake in *Mastigocladus* sp. strain CHP1, despite the fact that the cells are not able to switch quickly from one nitrogen source to the other. For instance, nitrate uptake was reduced by 89% in cultures previously grown on ammonium ($BG11_{NH4}$), possibly due to the lack of an active NRT substrate-binding protein (NrtA) needed to assimilate nitrate [47]. Likewise, ammonium uptake was reduced by 87% in cultures acclimated to nitrate ($BG11_{NO3}$). A preference for incorporation of particular nitrogenous substances has also been found for other thermal *Mastigocladus* spp. [30].

From the data obtained, it may be concluded that *Mastigocladus* sp. strain CHP1 displays great metabolic plasticity with respect to the usage of the nitrogen regimes offered. This genetic "versatility" may confer a great competitive advantage under natural conditions. Indeed, the importance of the members of *Stigonematales* as contributors of fixed nitrogen to the nitrogen cycle in the Porcelana hot spring was recently demonstrated by Alcamán et al. [2]. In addition, recent metatranscriptomic data from Porcelana microbial mats growing at 48 and 58 °C demonstrated that the *nif*H gene transcripts of *Mastigocladus* sp. strain CHP1 represented up to 87% of the total *nif*H gene transcripts in this hot spring. This further stresses the pivotal role of this specific strain in Porcelana, and potentially in other globally distributed hot springs where members of *Mastigocladus* dominate.

The findings of this study thus highlight the appropriateness of using *Mastigocladus* sp. strain CHP1 to answer existing questions concerning, for instance, the evolutionary strategies that enable extremophiles to evolve and functionally adapt in extreme environments. Specifically, such data may provide valuable insights into the potential use of thermophilic microorganisms with unique macromolecular properties and high metabolic rates for biotechnology.

Conclusions

Collectively, the data demonstrated that the CHP1 cyanobacterium strain isolated from the Porcelana hot spring phylogenetically belonged to an exclusive thermal clade within the Stigonematales. The strain was taxonomically identified as *Mastigocladus* sp. based on its morphological characters (uniseriate filaments and the true branching types T and Y). Its capacity to grow optimally at 50 °C demonstrated that *Mastigocladus* sp. strain CHP1 represented a moderately thermophilic cyanobacterium. Daily *nif*H gene expression patterns suggested different rhythmicity controls depending on temperature. High expression was found for the *gln*A and *nar*B genes in light, and the expression of these genes was shown to be differentially regulated by the nitrogen regime available. A clear preference for nitrate and a low tolerance for ammonium was observed. This work provides insights into the nitrogen acquisition behavior of *Mastigocladus* sp. strain CHP1, a major player in the nitrogen economy of the microbial mats in the Porcelana hot spring, revealing its physiological adaptations at high temperatures. Further analyses to better understand the unique genomic features and adaptation abilities that gave members of this clade the capacity to conquer these extremely hot environments are now warranted.

Acknowledgements

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Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2016.11.007.

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