Extremophiles (2008) 12:701–711
DOI 10.1007/s00792-008-0176-4

ORIGINAL PAPER

Identity and physiology of a new psychrophilic eukaryotic green alga, *Chlorella* sp., strain BI, isolated from a transitory pond near Bratina Island, Antarctica

Rachael M. Morgan-Kiss · Alexander G. Ivanov · Shannon Modla · Kirk Czymmek · Norman P. A. Hüner · John C. Priscu · John T. Lisle · Thomas E. Hanson

Received: 8 March 2008 / Accepted: 4 June 2008 / Published online: 26 July 2008

Abstract Permanently low temperature environments are one of the most abundant microbial habitats on earth. As in most ecosystems, photosynthetic organisms drive primary production in low temperature food webs. Many of these phototrophic microorganisms are psychrophilic; however, functioning of the photosynthetic processes of these enigmatic psychrophiles (the "photopsychrophiles") in cold environments is not well understood. Here we describe a new chlorophyte isolated from a low temperature pond, on the Ross Ice Shelf near Bratina Island, Antarctica. Phylogenetic and morphological analyses place this strain in the *Chlorella* clade, and we have named this new chlorophyte *Chlorella* BI. *Chlorella* BI is a psychrophilic species, exhibiting optimum temperature for growth at around 10°C. However, psychrophily in the Antarctic *Chlorella* was not linked to high levels of membrane-associated poly-unsaturated fatty acids. Unlike the model Antarctic lake alga, *Chlamydomonas raudensis* UWO241, *Chlorella* BI has retained the ability for dynamic short term adjustment of light energy distribution between photosystem II (PS II) and photosystem I (PS I). In addition, *Chlorella* BI can grow under a variety of trophic modes, including heterotrophic growth in the dark. Thus, this newly isolated photopsychrophile has retained a higher versatility in response to environmental change than other well studied cold-adapted chlorophytes.

Keywords Antarctica · *Chlorella* · Green alga · Photophysiology · Photopsychrophile

Abbreviations

A	Antheraxanthin
Chl-*a*	Chlorophyll a
Chl-*b*	Chlorophyll b
EPS	Epoxidation state of the xanthophyll cycle
$F_{685,695,715}$	77 K Fluorescence emission maxima at 685, 695, 715 nm
FAMEs	Fatty acid methyl esters
LHC II	Light harvesting II
PAR	Photosynthetically active radiation
PS I	Photosystem I
PS II	Photosystem II
PUFAs	Polyunsaturated fatty acids
T_{op}	Optimal growth temperature
T_{max}	Maximum growth temperature

Electronic supplementary material The online version of this article (doi:10.1007/s00792-008-0176-4) contains supplementary material, which is available to authorized users.

R. M. Morgan-Kiss (✉)
Department of Microbiology, Miami University,
700 E. High Street, Oxford, OH 45045, USA
e-mail: morganr2@muohio.edu

A. G. Ivanov · N. P. A. Hüner
Department of Biology and The Biotron,
University of Western Ontario, London,
ON N6A 5B7, Canada

S. Modla · K. Czymmek · T. E. Hanson
Delaware Biotechnology Institute, University of Delaware,
15 Innovation Way, Newark, DE 19711, USA

J. C. Priscu
Department of Land Resources and Environmental Sciences,
Montana State University, Bozeman, MT 59717, USA

J. T. Lisle
USGS Centre for Coastal and Watershed Research,
St Petersburg, FL 33701, USA

T. E. Hanson
College of Marine and Earth Studies,
University of Delaware, Newark, DE 19711, USA

V Violaxanthin
Z Zeaxanthin

Introduction

Environments where annual temperatures are permanently near or below the freezing point of water cover much of our planet (Priscu and Christner 2004). Organisms living in low temperature environments have evolved a number of adaptive mechanisms to counteract the effects of the cold such as, loss of membrane fluidity, decrease in enzymatic activity, and a decline in protein stability caused by cold temperatures (D'amico et al. 2006). A sub-set of organisms that exists within the psychrophilic group are the "photopsychrophiles" which rely on the photoautotrophic metabolic pathways to fix inorganic carbon using light energy (Morgan-Kiss et al. 2006). In addition to cold adaptation of general metabolic and cellular processes, these organisms must also balance temperature independent processes of light absorption and light energy transduction with downstream metabolic utilization of the light-derived energy products. Unlike rates of photochemical reactions, such as light absorption and charge separation, the rates of downstream metabolic reactions are extremely temperature sensitive. Imbalances between the production of stored energy products via photosynthetic electron transport and their utilization, can cause photo-oxidative damage. Mesophilic photoautotrophic organisms possess a variety of mechanisms to sense and respond to imbalances in "fast" photochemical reactions and "slow" temperature dependent processes of electron transport and downstream utilization of electron sinks through growth and metabolism, and thereby maintain balanced energy flow. These mechanisms can involve altering the capacity of the light harvesting antenna or changing sink capacity (Hüner et al. 1998).

Photosynthetic psychrophiles must possess regulatory mechanisms to stringently maintain a balance between energy absorbed and utilized. Although there are several reports on photosynthetic acclimation in photopsychrophiles (e.g., Cota 1985; Fiala and Oriol 1990; Lizotte and Priscu 1992; Devos et al. 1998; Mock and Hock 2005; Ralph et al. 2005), the adaptive mechanisms to the harsh polar environment are still poorly understood. The Antarctic green alga, *Chlamydomonas raudensis* UWO241 (Pocock et al. 2004), isolated from Lake Bonney located in the Transantarctic Mountains, is the best studied example. As a consequence of adaptation to a year-round stable environment of low temperatures (<5°C) and extreme shade caused by a 4 m thick permanent ice cover (including a 6 month period of no light), this photopsychrophile lacks several major acclimatory mechanisms in response to environmental change (Morgan-Kiss et al. 2002a, b, 2005; Szyszka et al. 2007). Thus, adaptation of this photoautotroph to the low temperature environment of a Dry Valley lake has resulted in a loss in the ability to adjust the light harvesting capacity of the antenna in response to an imbalance in the energy budget in this photopsychrophile. However, it is not known if the loss of light harvesting regulation by *C. raudensis* is representative of photopsychrophiles growing in other low temperature environments.

Bratina Island is located on the McMurdo Ice Shelf in the Ross Sea, and was formed by the deposition of sediment originating from the sea floor. During the summer months, the sediment enhances the warming effect of the sun and thousands of transitory ponds with vastly varying physical and chemical parameters form (Hawes and Howard-Williams 2003). These ponds are visually dominated by benthic-cyanobacterial mats. However, the mats exist as micro-oases for a variety of other autotrophic and heterotrophic bacteria as well as single-celled eukaryotes such as diatoms and chlorophytes which thrive within this micro-environment (Howard-Williams et al. 1989). Living mats in the ponds today are almost identical in structure to some of the oldest cyanobacterial mat fossils ever found, and it has been hypothesized that the biota inhabiting these ponds are living fossil examples of how life persisted in micro-refugia during periods of global glaciation (Vincent and Howard-Williams 2001).

Physical conditions and chemical properties vary widely from pond to pond. However, there are a number of highly variable and extreme environmental conditions common to all the ponds, such as high photosynthetically active radiation (PAR) and ultraviolet light (UV), total winter darkness, freeze/thaw cycles, and desiccation events (Hawes et al. 1999). This environment is in stark contrast with other Antarctic aquatic ecosystems, such as the stratified communities of the perennially ice-covered lakes of the McMurdo Dry Valleys, that are characterized by extremely stable year-round temperatures and consistently low PAR levels (Neale and Priscu 1995; Spigel and Priscu 1998; Fritsen and Priscu 1999). Here we report the discovery of a new chlorophyte species isolated from one of the transitory ponds, "Fresh Pond" (Hawes and Howard-Williams 2003), and describe the physiological consequences of adaptation to a highly variable/permanently low temperature environment. Based on phylogenetic and morphological information described below, we suggest the name *Chlorella* BI for this isolate.

Materials and methods

Site description, strain isolation and laboratory-controlled growth

A green alga was isolated from an algal mat residing in a transitory pond called "Fresh pond" located within an

ablation zone on the McMurdo Ice Shelf on the Ross Sea, south of Bratina Island (78°00′S, 165°35′E), Antarctica. The ablation zone is a 1,200 km^2 area of ice shelf that is covered with numerous transitory ponds colonized by thick cyanobacteria-dominated mats (Vincent et al. 1993). The green alga was originally observed as a green colony growing on plates of R2A at 4°C in the dark. The organism was subsequently purified to homogeneity by serial culturing on R2A agar plates.

Monocultures were grown in 100 mL batch cultures in either an organic carbon medium (R2A) (Reasoner and Geldreich 1985) or a salt medium (BG11) (Stanier et al. 1971) in culture tubes under continuous aeration and illumination in thermo-regulated aquaria as described by Morgan et al. (1998). Figure 1 shows the experimental setup for batch culturing under a stringently controlled temperature/light regime. Cultures were typically grown at a temperature/irradiance regime of 10°C/30 µmol photons m^{-2} s^{-1} unless otherwise indicated. Growth rates were calculated by monitoring the change in optical density at 750 nm (OD750) on a daily basis. Chlorophyll-a and -b contents were determined spectrophotometrically according to Jeffrey and Humphrey (1975).

Molecular analysis

Genomic DNA was isolated from mid-log cultures of the Antarctic green alga using a genomic DNA extraction kit (Fermentas, Hanover, MD, USA). A ~1,100 bp region of the nuclear 18S rRNA gene and a ~900 bp region of plastid 16S rRNA gene were amplified using the 519F/1406R universal primers (CAGCMGCCGCGGTAATWC/ACGGGCGGTGTGTRC, respectively) (Suzuki and Giovannoni 1996) which amplified both rRNA genes. The PCR products were gel purified, cloned into the pCR2.1-TOPO vector according to the manufacturer's instructions (Invitrogen), and sequenced at the University of Delaware Sequencing and Genotyping Center using standard primers and protocols (see http://www.udel.edu/dnasequence/UDSGC/Welcome.html for details). Alignment of DNA sequences was performed with ClustalW in Mega3.1 using 18S rRNA sequences of 1,146 bp and 16S rRNA sequences of 843 bp. Comparative sequences were selected from GenBank to represent major lineages of algae and eukaryotes. Closely affiliated sequences were identified by BLASTN searches. A neighbor-joining analysis was performed and bootstrap consensus trees (1,000 pseudoreplicates) were generated using the Kimura 2-parameter distance model (Kimura 1980) with pairwise gap deletion.

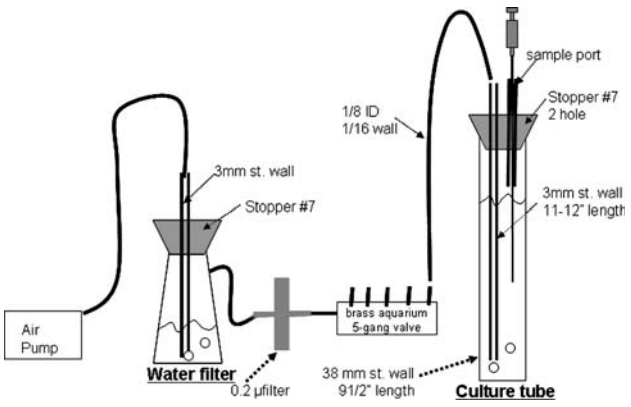

Fig. 1 Schematic of batch culture setup for growing 50–200 mL psychrophilic cultures under stringently controlled temperature/irradiance regime. Growth temperature is maintained by immersion of the culture tube in a thermo-regulated aquarium. Samples can be removed or supplements can be added axenically with a 9" needle attached to a syringe through a glass port in the rubber stopper

Pigment extraction and analysis

Total photosynthetic pigments were extracted from 1 mL of exponential-phase cultures grown either photoautotrophically (BG11 medium), mixotrophically (R2A medium + light) or heterotrophically (R2A medium + dark) under the standard growth regime. Cells were pelleted and pigments were extracted in 1 mL of 100% acetone for 1 h at 4°C in the dark. Extracts were overlayed with N_2 gas and stored at −20°C until use. Pigments were separated and quantified on a Dynamax Sperisorb C8 column (250 mm × 4.6 mm ID) with an attached guard column on a Class VP HPLC system (Shimadzu Scientific Instruments) and equipped with a column oven, UV/Vis, and fluorescence detectors according to the method of Van Heukelem and Thomas (2001). Pigments were detected using a measuring wavelength of 440 nm, and identified by retention times of mixed commercially available standards (DHI Water & Environment, Denmark), and quantified using a combination of purified standards and published extinction coefficients (Ston and Kosakowska 2002). The total pool sizes of the xanthophyll cycle pigments was calculated as a sum of violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z). The relative epoxidation state (EPS) of the xanthophyll cycle was calculated as (V + 0.5A)/(V + A + Z) (Demmig-Adams and Adams 1992).

Fatty acid methyl ester analysis

Total lipids were extracted from 5 mL of mid-log phase cultures grown in mixotrophic (R2A) media, and lipids were extracted with methanol:chloroform (2:1) from pelleted cells according to Bligh and Dyer (1959). Mass spectra were generated via collision-induced dissociation electrospray mass spectrometry on a VG Quattro (Department of Chemistry, University of Illinois at Urbana-Champaign) at a cone voltage of 90 V collecting data in

mass range of 100–500 m/z. Fatty acids were identified based on the molecular weight of known fatty acids (Sweetman et al. 1996) and by comparison with mass spectrum of phospholipids isolated from the photopsychrophile *C. raudensis*, whose fatty acid methyl ester (FAMEs) distribution was previously reported (Morgan-Kiss et al. 2002a).

FAMEs were extracted from a portion of the phospholipid samples via transesterification using the base-catalyzed method of Christie (2003) with minor modifications. Phospholipid samples were dried under N_2 and the pellet was dissolved in sodium methoxide and 5 μ Ci of [^{14}C]-methanol (American Radiolabelled Chemicals Inc, St Louis, MO, USA). This modification resulted in the production of FAMEs with a radiolabelled methyl group on the carboxy terminus of the methyl ester. FAMEs were extracted with petroleum ether and the entire sample was loaded on Analtech Silica Gel GHL plates impregnated with 20% $AgNO_3$. Plates were developed twice in 100% toluene at −20°C, and radiolabelled FAMEs were visualized by autoradiography.

77 K Fluorescence emission spectra

Low temperature (77 K) fluorescence emission spectra were collected from dark adapted fresh cultures on a PTI LS-100 luminescence spectrophotometer (Photon Technology International, South Brunswick, NJ, USA) equipped with a liquid nitrogen sampling device as described by Morgan et al. (1998). Samples were exposed to an excitation wavelength of either 436 or 472 nm to excite either Chl-*a* or Chl-*b*, respectively. Fluorescence spectra were recorded from 650 to 800 nm. All fluorescence spectra were corrected by subtracting the medium blank and were normalized to the PSII peak at 695 nm. Each spectrum represents the average of three scans. Identity of the fluorescence maxima was determined by Gaussian analysis (data not shown).

Electron microscopy

Mid-log phase cultures were pelleted, high pressure frozen with a Leica EMPACT high-pressure freezer, and freeze substituted in 4% OsO_4 in 100% acetone for 4 days at −80°C. Samples were warmed to −20°C for 4 h and then to 4°C for an additional 4 h. After 1 h at room temperature, they were dehydrated in 100% acetone and infiltrated with Spurr's resin (1:3, 1:1, 3:1. 100%; 1 h each). Following an overnight infiltration with 100% Spurr's, samples were embedded and polymerized at 60°C for 24 h.

Samples were sectioned on a Reichert-Jung Ultracut E ultramicrotome and collected onto formvar-carbon coated copper grids and stained with saturated uranyl acetate in methanol and Reynolds' lead citrate (Reynolds 1963). Samples were viewed with a Zeiss CEM 902 transmission electron microscope in brightfield mode at 80 kV. Images were recorded with an Olympus Soft Imaging System GmbH Megaview II digital camera.

Results

Phenotypic and phylogenetic analysis

Molecular phylogenies of *Chlorella* BI were inferred from partial sequences of the nuclear 18S and plastidic 16S rRNAs (Figs. 2, 3, respectively). Based on the nuclear rRNA gene, the isolate is closely affiliated with organisms in the *Chlorella* green algal clade (Fig. 2) with a bootstrap value of 100%. The closest matches to the Antarctic pond isolate were an environmental *Chlorella* isolate (*Chlorella* 2A3; AF357146) and *Micractinium pusillum* (AM231740) which were 98% identical to the *Chlorella* BI sequence over its entire length. Several Antarctic *Chlorella* 18S sequences were also included in this group; however, the newly isolated Antarctic chlorophyte exhibited comparable similarity between other Antarctic *Chlorella* strains and the type mesophilic species, *C. vulgaris*. Analysis of the plastid rRNA fragment also indicated that this alga is a member of the *Chlorella* clade (Fig. 3). There are no publicly available plastid 16S rRNA sequences from *Micractinium*,

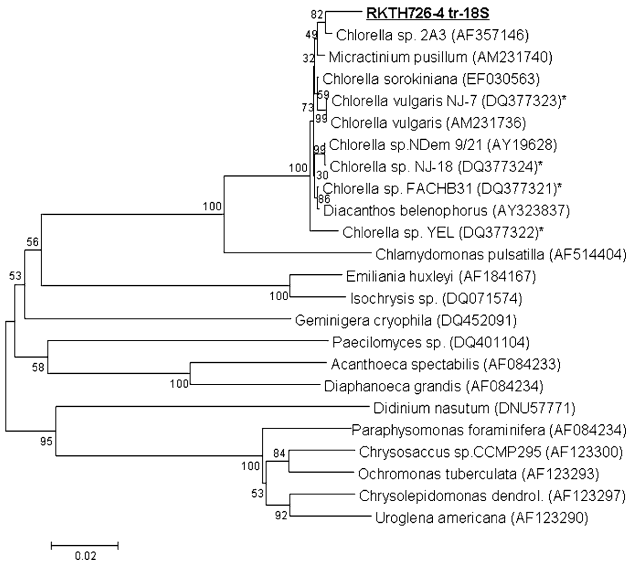

Fig. 2 Phylogenetic tree of *Chlorella* BI (RKTH726-tr-18S) and related species on the basis of 1,200 aligned nucleotide sites in 18S rDNA. Numbers are bootstrap values for 1,000 replicates using neighbor joining method. *Bar* is 0.02 substitutions per site. *Accession numbers* are indicated after each species name. *Asterisks* denote sequences from polar *Chlorella* spp

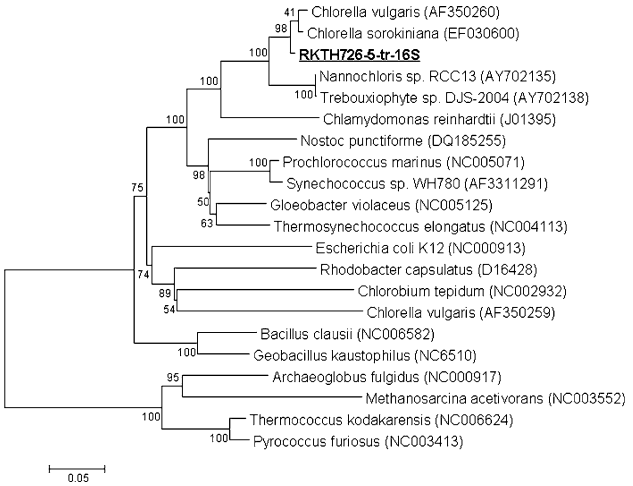

Fig. 3 Phylogenetic tree of *Chlorella* BI (RKTH726-5-tr-16S) based on plastid 16S rDNA sequences. *Numbers* are bootstrap values for 1,000 replicates using neighbor joining method. *Bar* is 0.05 substitutions per site. *Accession numbers* are indicated after each species name

precluding a more detailed comparison to this sub-group within the *Chlorella* clade.

Morphology of *Chlorella* BI was examined by transmission electron microscopy (Fig. 4) on mid-log phase culture samples grown under either mixotrophic or photoautotrophic conditions. Cells were spherical in shape, 3–5 μm in diameter, and primarily dispersed; although four-celled colonies were occasionally observed in cultures grown in either medium (data not shown). Cells grown under either condition exhibited a solitary chloroplast with a prominent pyrenoid body surrounded by multiple starch granules. The chloroplasts were relatively small, compared with those of mesophilic *Chlorella* sp. (Silverberg 1975). Many of the cells exhibited photosynthetic membranes (thylakoids) traversing the pyrenoid (Fig. 4a), a morphological characteristic common to the *Chlorella* clade. Cells grown in the organic medium exhibited more starch granules associated with the chloroplast as well as lipid storage bodies within the cytoplasm (Fig. 4b). Photosynthetic membranes were few in number and poorly organized, regardless of the growth medium (Fig. 4c). The cell walls were very pronounced, with numerous short fibrils (~ 100–200 nm in length) associated with the cell wall outer surface (Fig. 4d).

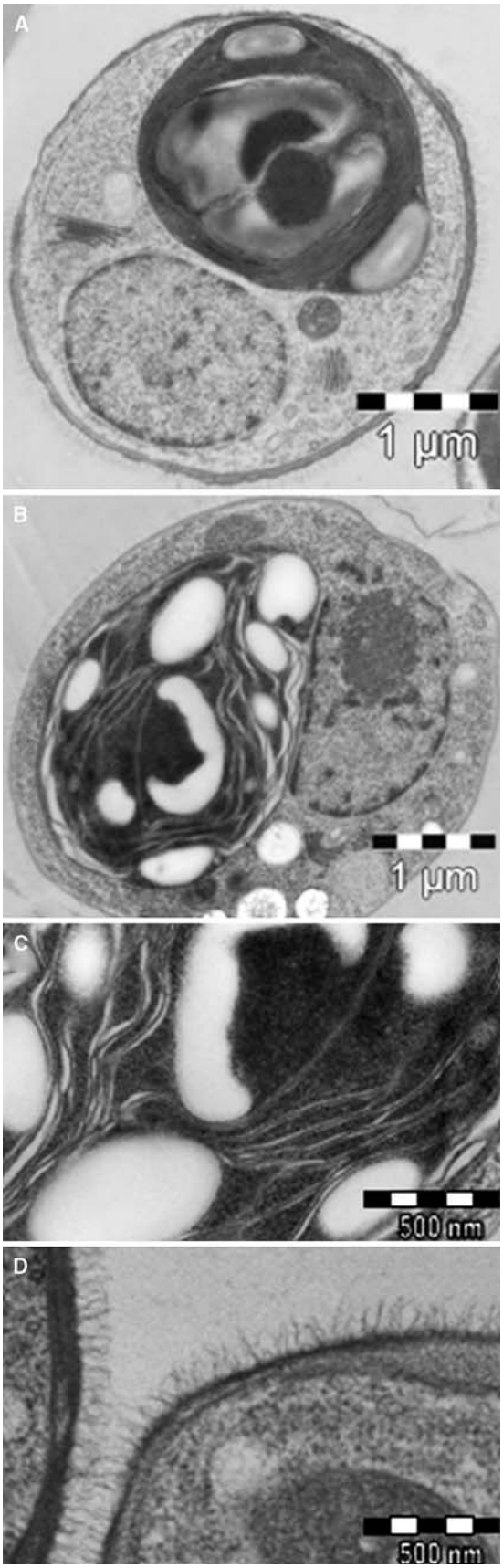

Fig. 4 Transmission electron micrographs of the Antarctic *Chlorella* BI grown under variable trophic modes. **a** Isolated cell from a culture grown under photoautotophic conditions. **b** Cell from a culture grown under mixotrophic growth conditions. **c** Thylakoid membranes and starch bodies in the chloroplast. **d** Cell wall decorated with fibrils

Growth phenotype and photosynthetic pigments

Batch cultures exhibited the highest growth rates when grown under mixotrophic conditions in the organic medium, R2A, with glucose as an organic carbon source (Table 1). Cultures also exhibited the ability to grow in the same medium heterotrophically in the dark, and photoautotrophically in an inorganic growth medium, BG11 (Table 1). Optimal growth temperature was approximately 10°C, and cultures were unable to grow at temperatures above 20°C, confirming the psychrophilic nature of *Chlorella* BI.

The Chl-*a:b* ratios estimated from extracts of exponential-phase cultures grown under either mixotrophic or photoautotrophic conditions were typical of those reported for mesophilic green alga. Cultures grown in the dark exhibited a reduction in the Chl a:b ratio (Table 1), likely caused by increased Chl-*b* levels (relative to Chl-*a*) estimated by HPLC methods (Table 2). Separation and identification of major photosynthetic pigments via HPLC indicated that this alga possesses a typical complement of carotenoids, and included xanthophyll cycle carotenoids violaxanthin (V), antheraxanthin (A) and zeaxanthin (Z), as well as neoxanthin and lutein (Table 2). Growth under either mixotrophic or photoautotrophic growth conditions had minimal effects on pigment distribution. However, heterotrophically grown cultures exhibited a reduction in the light harvesting carotenoid lutein (Table 2). Interestingly, the contribution of Z to the total xanthophylls pool size was relatively high (almost 50%) regardless of the growth conditions, which reflected in high efficiency of the xanthophyll cycle measured as lower EPS values (Table 2).

Table 1 Growth characteristics of *Chlorella* BI cultures grown in organic (R2A) versus inorganic (BG11) media at 10°C/30 µmol photons m^{-2} s^{-1}

Growth Media	Growth rate (day^{-1})	Doubling time (days)	Chl a:b
BG 11	0.33	2.08	3.53
R2A	0.44	1.56	3.34
R2A, dark	0.36	1.90	2.86
R2A *	0	NA	NA
R2A, dark*	0	NA	NA

To test for psychrophily, cultures were also grown at 20°C (*). The values represent the mean of two independent experiments

Table 2 Major photosynthetic pigments of *Chlorella* BI

Pigment	R2A	R2A, dark	BG11
Neoxanthin	14 ± 4	10 ± 4	14 ± 3
Violaxanthin	34 ± 2	21 ± 10	23 ± 6
Antheraxanthin	5 ± 4	8 ± 2	8 ± 4
Lutein	133 ± 9	85 ± 24	109 ± 22
Zeaxanthin	20 ± 4	15 ± 2	19 ± 3
Chlorophyll b	237 ± 9	439 ± 82	247 ± 12
V + A + Z	59	44	50
EPS	0.61	0.57	054

Pigments were extracted from whole cells isolated from exponentially growing cultures. Cultures were grown in either organic (R2A) or inorganic (BG11) medium at 10°C. Extracted pigments were separated via HPLC as described in "Materials and methods". The relative epoxidation state (EPS) of the xanthophyll cycle was calculated as (V + 0.5A)/(V + A + Z). Values represent µg pigment/mg Chl *a* ($n = 3$)

Fatty acyl methyl ester distribution

Overall desaturation as well as carbon chain length of FAMEs isolated from total membrane lipid pool of mid-log cultures grown under mixotrophic conditions was investigated by collision induced dissociation electrospray mass spectrometry and Argentation-TLC (Fig. 5; Table 3). A cone voltage of 90 V resulted in complete degradation of the phospholipids into the head groups and fatty acyl components (Fig. 5a). For TLC analysis, phospholipids were transesterified in the presence of [^{14}C]-labeled methanol to produce radiolabelled FAMEs, and FAMEs were separated by argentation TLC (Christie 2003). Migration was dependent upon the degree of unsaturation, with highly unsaturated methyl esters remaining closest to the origin (Fig. 5b). As a control, total phospholipids or radiolabelled fatty acyl methyl esters were also prepared from the psychrophilic *Chlamydomonas raudensis* UWO241, whose FAMEs distribution has been previously described, and was found to possess high levels of polyunsaturated FAMEs as well as relatively high levels of the

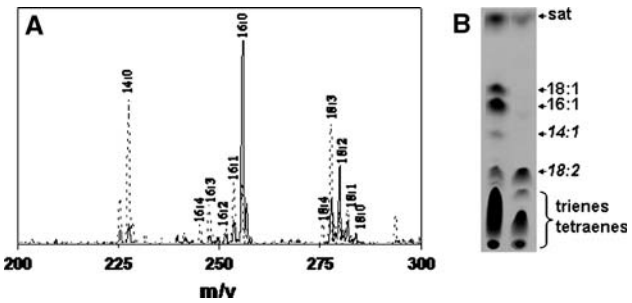

Fig. 5 Fatty acid composition of isolated membranes from the Antarctic *Chlorella* BI. **a** Distribution of fatty acids from total lipids isolated from *Chlorella* BI (*solid line*) and the Antarctic lake alga, *Chlamydomonas raudensis* (*dotted line*), as determined by negative ion mass spectrometry. The identity was determined by molecular weights of known fatty acids, and is denoted at the top of the peaks. **b.** Fatty acid composition as determined by argentation TLC of [C-14] labeled fatty acid methyl esters of the Antarctic lake alga *C. raudensis* (*left lane*) and *Chlorella* BI (*right lane*). FAMEs were radiolabelled by base-hydrolysis in the presence of [C-14]methanol as described in "Materials and methods"

short chain saturated fatty acid, myristate (14:0) (Morgan-Kiss et al. 2002a).

As expected, the analysis of isolated FAMEs in Antarctic lake psychrophile, *C. raudensis,* showed the presence of multiple PUFAs of the 16- and 18-carbon chain lengths (Fig. 5a; Table 3) as well as a relatively high level of FAMEs with low mobility on the TLC plate (Fig. 5b). In contrast with *C. raudensis*, the Antarctic pond alga exhibited relatively reduced levels of trienoic and tetraenoic FAMEs at the level of both the 16- and 18-carbon lengths, as well as low levels of the short chain saturated fatty acid, myristirate (14:0). The major unsaturated species in this Antarctica alga was a di-unsaturated 18-carbon FAME (Fig. 5a; Table 3). The reduced levels of these PUFAs were also confirmed by the lower levels of poorly migrating FAMEs on the TLC plate (Fig. 5b).

Table 3 Fatty acid distribution of cellular membranes isolated from *C. raudensis* and *Chlorella* BI

Fatty acid	Fatty acid distribution (percent of total by mass)	
	Chlorella BI	*C. raudensis*
14:0	3.48	25.86
16:0	49.88	11.15
16:1	5.18	11.09
16:2	2.01	2.91
16:3	1.77	7.11
16:4	0.38	3.55
18:0	0.54	0.63
18:1	5.88	6.39
18:2	19.25	5.88
18:3	11.50	21.59
18:4	0.12	3.83

FAMES were isolated from log phase cultures

Chlorophyll fluorescence

Low temperature (77 K) fluorescence emission spectra of whole cells of the psychrophilic alga were used to study in vivo the light energy distribution between the major photosynthetic complexes, which reflect the functional organization of the photosynthetic apparatus (Krause and Weis 1984). Cultures were grown under either photoautotrophic or mixotrophic conditions and the emission spectra from whole cells were collected under conditions that preferentially excite Chl-*a* (Fig. 6a, c) or Chl-*b* (Fig. 6b, d) in dark and light adapted samples.

Regardless of growth condition, *Chlorella* BI cells exhibited three well defined major chlorophyll fluorescence emission peaks centered at 685, 695–699 and 715 nm, which corresponded to the major pigment-protein complexes of light harvesting II (LHCII), photosystem II (PSII) and photosystem I (PSI) core complexes, respectively (Krause and Weis 1984) (Fig. 6). This 77 K fluorescence emission pattern is characteristic for green algae (Morgan et al. 1998; Sato et al. 2002). However, 77 K fluorescence spectra observed in some other psychrophilic algae

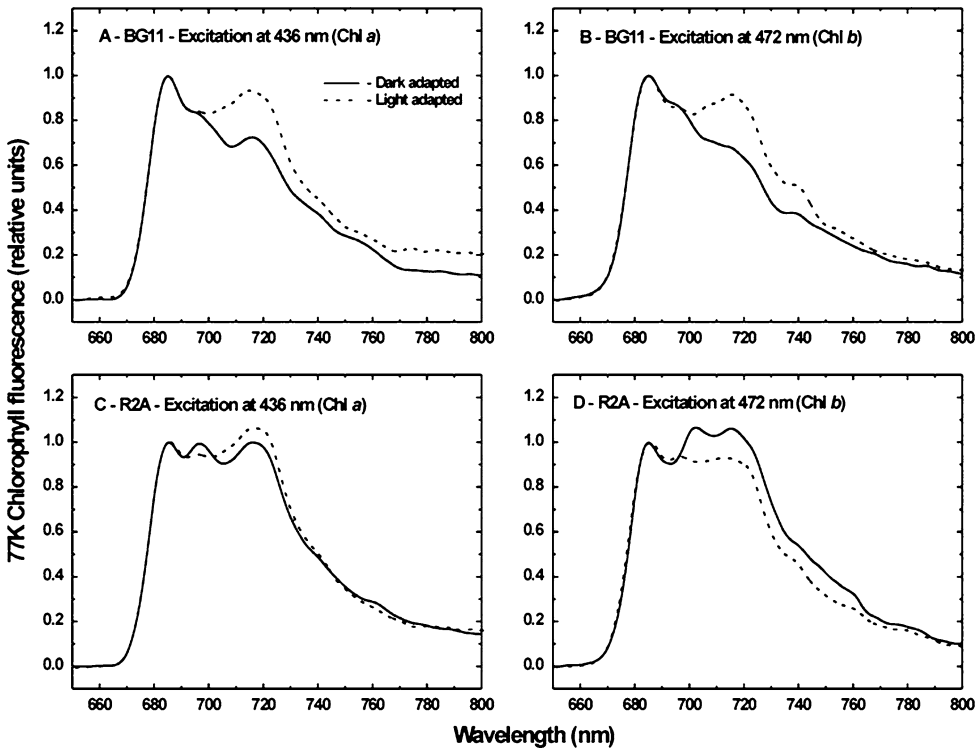

Fig. 6 Excitation energy distribution between photosystems as determined by low temperature (77 K) fluorescence emission. Cultures of the Antarctic *Chlorella* BI were grown under either photoautotrophic (BG11 medium; **a**, **b**) or mixotrophic (R2A medium; **c**, **d**). 77 K emission spectra from dark adapted (*solid lines*) and light adapted (*dashed lines*) whole cells of mid-log phase cultures was collected at an excitation wavelength that preferentially excited Chl-a (**a**, **c**) or *Chl-b* (**b**, **d**)

(including two Antarctic *Chlorella* sp.) tend to exhibit little to no fluorescence emission associated with PSI (Morgan et al. 1998; Erokhina et al. 2004).

While the peak positions corresponding to the major Chl–protein complexes between the samples were relatively constant, the peak intensities varied, depending on growth condition, dark or light acclimation, and excitation of either Chl-*a* or Chl-*b*. In cells grown under photoautotrophic conditions (BG 11 medium), the ratio of PSII(F_{695})- versus LHCII(F_{685})-emission remained steady regardless of the excitation wavelength or dark/light acclimation (Table 4). On the contrary, PSI- versus PSII-associated fluorescence ratios (F_{715}/F_{685}) were 26 and 34% higher under Chl*a* and Chl*b* excitation, respectively, in light acclimated compared to dark adapted samples (Table 4).

Cultures grown under mixotrophic conditions exhibited ~20% higher LHCII- versus PSII-associated fluorescence emission ratios under all conditions tested. The values of F_{715}/F_{685} ratios in dark adapted cells grown under mixotrophic conditions were 30% higher compared to photoautotrophically grown cells, independent of the excitation wavelength (Table 4). Moreover, light acclimated mixotrophic cells did not exhibit any significant light-dependent increase in the relative intensity of PSI peak (F_{715}) (Fig. 6) and F_{715}/F_{685} ratios (Table 4).

Discussion

Permanently low temperature environments are abundant on our planet and are typically dominated by microbial life, where photoautotrophic microbes are the dominant primary producers (Laybourn-Parry and Pearce 2007). *Chlorella* BI is a new chlorophyte species isolated from the unique and dynamic low temperature environment of an algal mat residing in an Antarctic transitory pond.

Table 4 Low temperature (77 K) fluorescence emission ratios of dark adapted (D) and light acclimated (L) *Chlorella* BI cells grown in inorganic (BG11) and organic (R2A) media

Sample	$F_{685}/F_{694–699}$	F_{715}/F_{695}
BG 11, exc. 436 (D)	0.829	0.732
BG 11, exc. 436 (L)	0.840	0.929
BG 11, exc. 472 (D)	0.871	0.681
BG 11, exc. 472 (L)	0.860	0.913
R2A, exc. 436 (D)	0.990	1.000
R2A, exc. 436 (L)	0.940	1.050
R2A, exc. 472 (D)	1.060	1.050
R2A, exc. 472 (L)	0.932	0.927

77 K emission spectra were excited at 436 nm (Chl-*a*) or 472 nm (Chl-*b*). F_{685}, F_{695} and F_{715} represent the peak emission intensities of the major Chl-protein complexes of LHCII, PSII and PSI, respectively. The values are averages from three replicates

Phylogenetic evidence on the basis of nuclear 18S and plastid 16S rDNA sequence analysis confirms that this green alga belongs to the *Chlorella* clade within the Chlorellaceae family. This clade contains both the "true" *Chlorella* species, and a number of other species that cluster within the clade, but are morphologically distinct. On the basis of SSU rRNA analysis, three "true" *Chlorella* species have been suggested: *C. vulgaris*, *C. lobophora*, and *C. sorokiniana* (Krienitz et al. 2004). These species are morphologically similar and exhibit spherical, solitary cells with no mucilage or cell wall decorations. In contrast, some studies have shown that the morphologically distinct genera such as *Micractinium* and *Actinastrum* are also part of the *Chlorella* clade (Krienitz et al. 2004; Luo et al. 2006).

While several species of the genus *Micractinium* have been described, only one strain, *M. pusillum,* is currently available as a pure monoculture. Based on 18S rDNA, the Antarctic pond alga was more closely related to *Micractinium pusillum* than the "true" *Chlorella* sp. *C. vulgaris* and *C. sorokiniana*. However, because of the limited availability of other *Micractinium* sequences, including the absence of publicly available plastid 16S rDNA for this genus, we cannot definitively conclude that *Chlorella* BI is a new *Micractinium* species.

The morphology of *Chlorella* BI indicates that this strain is part of the *Chlorella* clade, but it is likely not included within the "true" *Chlorella* group. The strain displayed traits characteristic of the *Chlorella* clade as well as the "true" *Chlorella* species including dispersed spherical cells in liquid culture with pyrenoids transversed by thylakoid membranes. However, *Chlorella* BI exhibited a pronounced cell wall decorated with fibrils. While *Micractinium* species exhibit cell wall decorations such as spines and bristles, these are much more pronounced and fewer in number than exhibited by *Chlorella* BI (Luo et al. 2006). Thus, the fibril morphology of *Chlorella* BI could represent a novel morphological feature and may suggest that this strain represents a new group within the *Chlorella* clade. Resolution of the taxonomic position of this isolate will require further phylogenetic data.

Cultures of the *Chlorella* BI exhibited a dependence on low growth temperatures (T_{op} ~10°C), and failed to grow at temperatures above 20°C, thereby classifying it as a true psychrophile, according to the definition of Morita (1975). Photoautotrophic growth rates were comparable with that of other psychrophilic phototrophs (Morgan et al. 1998; McLachlan et al. 1999). In *C. raudensis*, psychrophily has been linked to the presence of the short chain fatty acid, myristate, as well as high levels of polyunsaturated fatty acids (PUFAs) in its membrane lipids (Morgan-Kiss et al. 2002a). *Chlorella* BI possesses neither a high level of myristate, nor the PUFAs, with a fatty acyl content similar to other mesophilic *Chlorella* sp., such as relatively high levels

of a di-unsaturated 18-C FATM (Chen and Johns 1991). Since adjustments in membrane fluidity and lowering the lipid transition temperature (T_o) are a primary adaptive mechanisms to life at low temperatures (Nishida and Murata 1996), *Chlorella* BI may utilize other mechanisms to maintain a low T_o, such as altering the membrane protein:lipid ratio or adjusting lipid head group class distribution (Gushina and Harwood 2006). Alternatively, *Chlorella* BI may be a moderate psychrophile relative to *C. raudensis*, and the moderate levels of unsaturated FAMEs observed could be sufficient for low temperature adaptation in this Antarctic chlorophyte. Supporting this notion *Chlorella* BI remains viable after long term exposure to temperatures above T_{max}, while *C. raudensis* do not survive under the same treatment (R. Morgan-Kiss, unpublished results).

Chlorella BI exhibited a dependence on an organic carbon source (glucose) for maximum growth rates and dark heterotrophic growth rates were comparable with photoautotrophic conditions (Table 1). Both traits have been observed in the mesophilic *Micractinium pusillum* (Bouarb et al. 2004), as well as some *Chlorella* sp. (Pulich and Ward 1973). Reduced growth rates in the absence of an organic carbon source may reflect a reduced chloroplast and poorly developed light harvesting membrane system indicated by relatively disorganized thylakoids in the chloroplast of *Chlorella* BI. The organic rich/highly shaded environment (Stal 1995) in the interior of microbial mats likely provided the selective force for such adaptations, which could provide significant advantages during the Antarctic shoulder seasons when incident irradiation intensity is particularly low.

Chlorella BI exhibited the ability to alter its photosynthetic apparatus in response to prevailing conditions. This strongly contrasts with *C. raudensis*, which has lacks such capacity. At the level of pigment composition, cells exhibited a pigment complement typical of green algal species, including xanthophyll cycle pigments violaxanthin, antheraxanthin, and zeaxanthin. The xanthophyll cycle protects the light harvesting apparatus from light absorption and by dissipating of excess energy. This ability would be advantageous in the variable light environment experienced by algae residing in the Antarctic transitory ponds (Vincent et al. 1993), particularly if cells were exposed at the mat surface.

At the level of dynamic energy distribution between photosystems, 77 K fluorescence emission spectra clearly demonstrate that PSI/PSII stoichiometry and/or distribution of the light excitation energy between PSII and PSI of cells grown under mixotrophic (R2A medium, light) conditions differ from these in photoautotrophic cells (Fig. 6). Under strictly photoautotrophic growth, the organization of the photosynthetic apparatus favored excitation of PSII (Fig. 6a), which is indicative of a relatively oxidized photosynthetic electron transport system. However, when cells were grown in the light in the presence of an organic carbon source, an up-regulation of PSI fluorescence peak was observed (Fig. 6c), resulting in 30% higher F_{715}/F_{685} ratio (Table 4). This implies that the photosynthetic apparatus under mixotrophic conditions is altered to favor excitation of PSI. Such an effect is typically observed in cells where the photosynthetic electron pool is in a relatively reduced state. Similar responses of PSI/PSII to trophic status are typical of mesophilic algae (Kovacs et al. 2000). In addition, light acclimated photoautotrophically grown cells exhibited an increased of PSI fluorescence peak, which resulted in higher F_{715}/F_{685} ratio compared to dark adapted cells (Table 4). Interestingly, this light dependent adjustment of the energy distribution between PSII and PSI was not observed in cells grown under mixotrophic conditions. Thus, *Chlorella* BI dynamically regulates the distribution of excitation energy between the two photosystems in response to both light and trophic status. This ability contrasts with observations of the psychrophile, *C. raudensis,* which has lost the ability to functionally regulate photosynthetically-derived energy distribution via this mechanism, as a consequence of adaptation to an extremely stable low temperature and low light environment (Morgan-Kiss et al. 2002b). We hypothesize that the complex photoadaptive behavior displayed by *Chlorella* BI reflects the need to survive the highly variable light environment of Antarctic transitory ponds.

The isolation and characterization of *Chlorella* BI has improved our understanding of primary productivity in the transitory ponds of the Ross Ice Shelf, where microbial communities are exposed to permanent cold but otherwise highly variable light, salinity and freeze-thaw regimes. *Chlorella* BI will provide an important model system for comparative studies with *C. raudensis*, isolated from the more stable Dry Valley Lakes ecosystem, to examine the full diversity of adaptive strategies that allow these organisms to thrive in permanently cold environments.

Acknowledgments The authors would like to thank Bruce Kingham at the University of Delaware Sequencing facility for sequencing reactions. We would also like to thank personnel at The Mass Spectrometry Facility at the University of Illinois Urbana-Champaign for training and advice regarding the Quattro MS. This work was supported in part through grants from the National Science and Engineering Council of Canada (NPAH & AGI) and the National Science Foundation grants MCB 0237335 (TEH) & NSF-OPP 0631494 (JCP & RMK).

References

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917

Bouarb L, Dauta A, Loudiki M (2004) Heterotrophic and mixotrophic growth of *Micractinium pusillum* Fresenius in the presence of

acetate and glucose: effect of light and acetate gradient concentration. Wat Res 38:2706–2712

Chen F, Johns MR (1991) Effect of C/N ratios and aeration on the fatty acid composition of heterotrophic *Chlorella sorokiniana*. J Appl Phycol 3:203–209

Christie WW (2003) Lipid analysis: isolation, separation, identification and structural analysis of lipids, 3rd edn, vol 15. The Oily Press, Bridgewater

Cota GF (1985) Photoadaptation of high arctic ice algae. Nature 315:219–222

D'amico S, Collins T, Marx JC, Feller G, Gerday C (2006) Psychrophilic microorganisms: challenges for life. EMBO Rep 7:385–389

Demmig-Adams B, Adams WWIII (1992) Photoprotection and other responses of plants to high light stress. Ann Rev Plant Physiol Plant Mol Biol 43:599–626

Devos N, Ingouff M, Loppes R, Matagne R (1998) RUBISCO adaptation to low temperatures: a comparative study in psychrophilic and mesophilic unicellular algae. J Phycol 34:665–660

Erokhina LG, Shatilovich AV, Kaminskaya OP, Gilichinskii DA (2004) Spectral properties of ancient green algae from Antarctic Dry Valley permafrost. Microbiology 73:485–487

Fiala M, Oriol L (1990) Light-temperature interactions on the growth of Antarctic diatoms. Mar Chem 35:169–177

Fritsen CH, Priscu JC (1999) Seasonal change in the optical properties of the permanent ice cover on Lake Bonney, Antarctica: consequences for lake productivity and dynamics. Limnol Oceanogr 44:447–454

Gushina IA, Harwood JL (2006) Mechanisms of temperature adaptation in poikilotherms. FEBS Lett 580:5477–5483

Hawes I, Howard-Williams C (2003) Pond life on the McMurdo Ice Shelf, one of the world's strangest ecosystems. NIWA 11:21–22

Hawes I, Smith REH, Howard-Williams C, Schwarz A-M (1999) Environmental conditions during freezing, and response of microbial mats in ponds of the McMurdo Ice Shelf, Antarctica. Antarc Sci 11:198–208

Howard-Williams C, Pridmore R, Downes MT, Vincent WF (1989) Microbial biomass, photosynthesis and chlorophyll a related pigments in the ponds of the McMurdo Ice shelf, Antarctica. Antarc Sci 1:125–131

Hüner NPA, Öquist G, Sarhan F (1998) Energy balance and acclimation to light and cold. Trends Plant Sci 3:224–230

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophyll a, b, c1, c2 in higher plants, algae and natural phytoplankton. Biochem Physiol Pflanz 167:191–194

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Molec Evol 16:111–120

Kovacs L, Wiessner W, Kis M, Nagy F, Mende D, Demeter S (2000) Short- and long-term redox regulation of photosynthetic light energy distribution and photosystem stoichiometry by acetate metabolism in the green alga, *Chlamydobytrys stellata*. Photosyn Res 65:231–247

Krause GH, Weis E (1984) Chlorophyll fluorescence as a tool in plant physiology. II. Interpretation of fluorescence signals. Photosyn Res 5:139–157

Krienitz L, Hegewald E, Hepperle D, Huss VAR, Rohr T, Wolf M (2004) Phylogenetic relationship of *Chlorella* and *Parachlorella* gen. nov. (Chlorophyta, Trebouxiophyceae). Phycolog 43:529–542

Laybourn-Parry J, Pearce DA (2007) The biodiversity and ecology of Antarctic lakes: models for evolution. Philos Trans R Soc Lond B Biol Sci 362:2273–2289

Lizotte MP, Priscu JC (1992) Photosynthesis-irradiance relationships in phytoplankton from the physically stable water column of a perennially ice-covered lake (Lake Bonney, Antarctica). J Phycol 28:179–185

Luo W, Pflugmacher S, Proschold T, Walz N, Krienitz L (2006) Genotype versus phenotype variability in *Chlorella* and *Micractinium* (Chlorophyta, Trebouxiophyceae). Protist 157:315–333

McLachlan JL, Curtis JM, Keusgen M, Boutilier K, Keusgen M, Seguel MR (1999) *Tetreutreptia pomquetensis* (Euglenophyta), a psychrophilic species: growth and fatty acid composition. J Phycol 35:280–286

Mock T, Hock H (2005) Long-term temperature acclimation of photosynthesis in steady-state cultures of the polar diatom *Fragilariopsis cylindrus*. Photosyn Res 85:307–317

Morgan RM, Ivanov AG, Priscu JC, Maxwell DP, Hüner NPA (1998) Structure and composition of the photochemical apparatus of the Antarctic green alga, *Chlamydomonas subcaudata*. Photosyn Res 56:303–314

Morgan-Kiss R, Ivanov AG, Williams J, Mobashsher K, Hüner NP (2002a) Differential thermal effects on the energy distribution between photosystem II and photosystem I in thylakoid membranes of a psychrophilic and a mesophilic alga. Biochim Biophys Acta 1561:251–265

Morgan-Kiss RM, Ivanov AG, Hüner NPA (2002b) The Antarctic psychrophile, *Chlamydomonas subcaudata,* is deficient in state I–state II transitions. Planta 214:435–445

Morgan-Kiss RM, Ivanov AG, Pocock T, Król M, Gudynaite-Savitch L, Hüner NPA (2005) The Antarctic psychrophile, *Chlamydomonas raudensis* Ettl (UWO241) (CHLOROPHYCEAE, CHLOROPHYTA) exhibits a limited capacity to photoacclimate to red light. J Phycol 41:791–800

Morgan-Kiss RM, Priscu JP, Pocock T, Gudynaite-Savitch L, Hüner NPA (2006) Adaptation and acclimation of photosynthetic microorganisms to permanently cold environments. Microbiol Molec Biol Rev 70:222–252

Morita RY (1975) Psychrophilic bacteria. Bacteriol Rev 39:144–167

Neale PJ, Priscu JC (1995) The photosynthetic apparatus of phytoplankton from a perennially ice-covered Antarctic lake: acclimation to an extreme shade environment. Plant Cell Physiol 36:253–263

Nishida I, Murata N (1996) Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids. Ann Rev Plant Physiol Plant Mol Biol 47:541–568

Pocock T, Lachance M-A, Proschold T, Priscu JC, Kim S, Huner NPA (2004) Identification of a psychrophilic green alga from Lake Bonney Antarctica: *Chlamydomonas raudensis* ETTL. (UWO 241) (*Chlorophyceae*). J Phycol 40:1138–1148

Priscu JC, Christner BC (2004) Earth's Icy Biosphere. In: Bull AT (ed) Microbial diversity and bioprospecting. ASM Press, Washington, D.C., pp 130–145

Pulich WM, Ward CH (1973) Physiology and ultrastructure of an oxygen-resistant *Chlorella* mutant under heterotrophic conditions. Plant Physiol 51:337–334

Ralph PJ, McMin A, Ryan KG, Ashworth C (2005) Short-term effect of temperature on the photokinetics of microalgae from the surface layers of Antarctic pack ice. J Phycol 41:763–769

Reasoner DJ, Geldreich EE (1985) A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol 49:1–7

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–212

Sato A, Kurano N, Senger H, Miyachi S (2002) Regulation of energy balance in Photosystems in response to changes in CO_2 concentrations in light intensities during growth in extremely-high-CO_2-tolerant green microalgae. Plant Cell Physiol 43:440–451

Silverberg BA (1975) An ultrastructural and cytochemical characterization of microbodies in the green algae. Protoplasma 83:269–295

Spigel RH, Priscu JC (1998) Physical limnology of the McMurdo Dry Valley lakes. In: Priscu JC (ed) Ecosystem dynamics in a Polar

Desert: the McMurdo Dry valleys, Antarctica Antarctic Research Series, vol 72. American Geophysical Union

Stal LJ (1995) Physiological ecology of cyanobacteria in microbial mats and other communities. New Phytol 131:1–32

Stanier RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae. Bacteriol Rev 35:171–205

Ston J, Kosakowska A (2002) Phytoplankton pigments designation-an application of RP-HPLC in qualitative and quantitative analysis. J Appl Phycol 14:205–210

Suzuki MT, Giovannoni SJ (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 62:625–630

Sweetman G, Trinei M, Modha J, Kusel J, Freestone P, Fishov I, Joseleau-Petit D, Redman C, Farmer P, Norris V (1996) Electrospray ionization mass spectrometric analysis of phospholipids of *Escherichia coli*. Molec Microbiol 20:233–238

Szyszka B, Ivanov AG, Huner NPA (2007) Psychrophily induces differential energy partitioning, photosystem stoichiometry and polypeptide phosphorylation in *Chlamydomonas raudensis*. Biochim Biophys Acta 1767:789–800

Van Heukelem L, Thomas C (2001) Computer-assisted high-performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. J Chromatog A 910:31–49

Vincent WF, Downes MT, Castenholz R, Howard-Williams C (1993) Community structure and pigment organisation of cyanobacteria-dominated microbial mats in Antarctica. Eur J Phycol 28:213–221

Vincent WF, Howard-Williams C (2001) Life on snowball Earth. Science 287:2421