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ORIGINAL PAPER

Temperature responses of growth, photosynthesis, fatty acid and nitrate reductase in Antarctic and temperate *Stichococcus*

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Abstract *Stichococcus*, a genus of green algae, distributes in ice-free areas throughout Antarctica. To understand adaptive strategies of *Stichococcus* to permanently cold environments, the physiological responses to temperature of two psychrotolerants, *S. bacillaris* NJ-10 and *S. minutus* NJ-17, isolated from rock surfaces in Antarctica were compared with that of one temperate *S. bacillaris* FACHB753. Two Antarctic *Stichococcus* strains grew at temperature from 4 to 25°C, while the temperate strain could grow above 30°C but could not survive at 4°C. The photosynthetic activity of FACHB753 at lower than 10°C was less than that of Antarctic algae. Nitrate reductase in NJ-10 and NJ-17 had its optimal temperature at 20°C, in comparison, the maximal activity of nitrate reductase in FACHB753 was found at 25°C. When cultured at 4–15°C a large portion of unsaturated fatty acids in the two Antarctic species was detected and the regulation of the degree of unsaturation of fatty acids by temperature was observed only above 15°C, though the content of the major unsaturated fatty acid αC18:3 in FACHB753 decreased with the temperatures elevated from 10 to 25°C. Elevated nitrate reductase activity and photosynthetic rates at low temperatures together with the high proportion of unsaturated fatty acids contribute to the ability of the Antarctic *Stichococcus* to thrive.

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Introduction

Antarctica is the coldest area on earth with an average monthly temperature from −10 to −30°C in winter and around 0°C in summer and it has the lowest temperature record of −89.5°C. In general, the water temperature in Antarctic ranges from −1.8 to 5.0°C no matter in winter or in short summer (Wiencke and Dieck 1990). The freshwater Antarctic phytoplanktons are able to grow under the constant low temperatures from 0 to 5.0°C (Priddle et al. 1986). Except psychrophiles, which could not survive above 15°C, many Antarctic phytoplanktons exhibit the ability to grow at temperatures above 20°C (Seaburg et al. 1981; Hu et al. 2008). These psychrotolerants are often subjected to the ambient environment with temperatures much lower than the optimal growth temperature. A long-term exposure to the extremely low temperature may induce corresponding adaptation of morphology, ultrastructure, physiology, biochemical composition and gene expression (Nagashima et al. 1995; Teoh et al. 2004; Stibal and Elster 2005; Hu et al. 2008; Li et al. 2009; Lu et al. 2009). Many concerns have been concentrated on the polar psychrophiles and few reports on the psychrotolerants can be found. In comparison with mesophiles, the content of the poly-unsaturated and short chain fatty acids in the membrane lipid is much higher, the optimal enzyme activity of nitrate reductase, glutathione reductase, argininosuccinate lyase shifts toward lower temperatures and rates of photosynthesis and respiration show a higher value in polar psychrophiles (Vona et al. 2004; Di Martino Rigano et al. 2006; Morgan-Kiss et al. 2006; Ding et al.

2007). In contrast, Morgan-Kiss et al. (2008) found that the composition of the membrane lipid in psychrophilic *Chlorella* BI was similar to that in mesophilic one and no short chain fatty acids were detected.

Stichococcus Nägeli, a genus of green algae, which includes about 14 species, dwells mostly in moist soil, on the wall, the trunk and aquatic plants (Ettl and Gärtner 1995; Handa et al. 2003; Neustupa et al. 2007). *Stichococcus bacillaris*, the model species of this genus, distributes all over the world including Antarctica, showing an extensive adaptability to changing temperatures, salinities and pH values with short life cycles (Pollio et al. 1997). Moreover, *Stichococcus* species have been identified to be a promising alternative feedstock for biodiesel production owing to the high lipid content together with high growth rate and biomass (Olivieri et al. 2011). Many species of genus *Stichococcus* have been isolated from Antarctica, which shows that these show good adaptation to the extremely low temperature (Vinocur and Izaguirre 1994; Broady 1996; McKnight et al. 2000; Massalski et al. 2001; Teoh et al. 2004; Hughes 2006). Study on the temperature responses of *Stichococcus* will facilitate the understanding of its wide distribution in Antarctica. In this study, we isolated two unicellular green alga strains, NJ-10 and NJ-17, from the surface of Antarctic rock. Based on the morphology, ultrastructure and 18S rDNA sequence analysis, they belong to the genus *Stichococcus*, one is *S. bacillaris* and the other is *S. minutus*. We compared the temperature responses of these two Antarctic *Stichococcus* with the temperate *S. bacillaris* FACHB753 and analyzed their cold tolerance.

Materials and methods

Isolation

Samples were scraped from the surface of wet rocks near the Zhongshan Station (69°22′S–76°22′E) of Antarctica in January 1999. In the laboratory, they were cultured in BG11 liquid medium (Stanier et al. 1971) at 4°C and 50 μmol photons m^{-2} s^{-1} for approximately 2 weeks. Then individual *Stichococcus* cells were isolated with micropipettes after a series of dilutions of the culture. *S. bacillaris* NJ-10 and *S. minutus* NJ-17 isolated were further purified by repeated streaking on BG11 medium solidified with 2% agar. *S. bacillaris* FACHB753 was purchased from the FACHB-Collection (Freshwater Algae Collection of the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China). The algal strains were cultured in BG11 medium at 4°C under continuous illumination with an intensity of 50 μmol photons m^{-2} s^{-1} provided by cool-white fluorescent lamps (Philips 40 W).

Light and electron microscopy

Cells in logarithmic growth phase (4°C) were observed and photographed under the Olympus BX41 microscope (Olympus, Tokyo, Japan) for light microscopy. For electron microscopy, cells were firstly fixed using glutaraldehyde (5%, v/v) in 0.2 M sodium cacodylate buffer (pH 7.2) containing 6% (w/v) sucrose for 2 h, and then fixed in 2% OsO_4 for 1 h. After that, cells were dehydrated in a series of gradient ethanol and embedded in Spurr's resin. Ultrathin sections obtained by a diamond knife were double-stained with 2% aqueous uranyl acetate and lead citrate. Finally, specimens were examined by the Hitachi H-7000FA transmission electron microscope (Hitachi, Tokyo, Japan).

Molecular phylogenetics

The total genomic DNA of the algae was extracted according to the manufacturer's instructions of a glass milk DNA isolation kit (Fermentas, Vilnius, Lithuania). Polymerase chain reaction (PCR) was performed using the general primers 18S-1 (5′-tggttgatcctgccagtagtc-3′) and 18S-2 (5′-tgatccttctgcaggttcacc-3′) to amplify 18S rDNA gene as previously described (Hu et al. 2008). The PCR products were gel purified and cloned into pMD18-T (Takara, Dalian, China) for sequencing. Manipulations of DNA were performed according to the standard methods. *Stichococcus* species included in phylogenetic analysis were selected as described by Neustupa et al. (2007). Phylogenetic analyses were performed by PAUP4.0b (Swofford 1998), with 1,000 bootstrap replicates for neighbor-joining and parsimony analyses, 100 replicates for the maximum likelihood analysis using *Lobosphaera tirolensis* (AB006051) and *Coenocystis inconstans* (AB017435) as the outgroup. Genbank number of *S. bacillaris* NJ-10, *S. minutus* NJ-17 and *S. bacillaris* FACHB753 were JN400255, JN400256 and EU045358, respectively.

Growth at various temperatures

Stichococcus cells were grown in 300-ml polycarbonate flasks containing 100 ml BG11 liquid medium (the starting optical density $OD_{730} = 0.05$) at six different temperatures: 4, 10, 15, 20, 30 and 35°C under continuous illumination with the intensity of 100 μmol photons m^{-2} s^{-1} in static culture, and each was performed in triplicate. Cell density was monitored at 730 nm daily, and specific growth rates (μ) were calculated with the equation $\mu = (\ln X_t - \ln X_0)/t$, in which X_0 is the initial cell density and X_t is the cell density after t days.

Freeze tolerance characteristics

S. bacillaris NJ-10 and *S. minutus* NJ-17 grown at 4, 10, 15 and 25°C in logarithmic growth phase were harvested by centrifugation and cell pellets were frozen at −20°C in the dark for 8 days. The frozen-thawed cell pellets were resuspended in fresh BG11 media, diluted to $OD_{730} = 0.04$ and cultivated in the test tube for 12 days at 4°C under continuous illumination with the intensity of 100 μmol photons m^{-2} s^{-1}, and each was performed in triplicate.

Photosynthesis and respiration versus temperature responses

Cells grown at 10°C in logarithmic growth phase were collected by centrifugation, resuspended in a fresh BG11 medium (supplemented with 10 mM $NaHCO_3$) and transferred to the Clark oxygen electrode (Hansatech Instruments Ltd., Norfolk, UK) chamber. Photosynthesis under the saturation intensity (about 700 μmol photons m^{-2} s^{-1}) and respiration versus temperature responses were measured at 5, 10, 15, 20, 25, 30 and 35°C, respectively.

Nitrate reductase assay

Algal cells grown at 10°C in logarithmic growth phase were harvested by centrifugation (6,000*g*, 5 min). Cell pellets were washed by 0.1 M Tris–HCl buffer (pH 7.5) twice, placed in liquid nitrogen for a quick frozen and grinded fully, then, protein extraction buffer (0.1 M Tris–HCl, pH 7.5, 0.3 mM Na_2EDTA, 2 mM DTT) was added. Supernatants as the crude enzyme were collected after centrifugation at 4°C, 12,000*g* for 20 min. Nitrate reductase activity was assayed in 2 ml reaction mixture containing 1.2 ml 0.1 M KNO_3, 0.4 ml 2 g l^{-1} NADH and 0.4 ml crude enzyme. The reaction was started by addition of the electron donor NADH. The assays were carried out at different temperatures for 30 min with triplicate reactions and stopped by the addition of 0.5 ml of 1 M zinc acetate solution plus 0.5 ml absolute ethanol, then the tubes were quickly shaken, centrifuged at 4°C, 6,000*g* for 5 min and the nitrite content in the supernatant was estimated at 540 nm with the addition of 1 ml 1% (w/v) sulfanilamide and 1 ml 0.02% (w/v) *N*-(1-Naphthyl) ethylenediamine dihydrochloride at 25°C after 30 min. Meanwhile, protein concentration in crude enzyme was estimated by the method of Bradford. Nitrate reductase activity was expressed as nmol nitrite produced min^{-1} mg^{-1} protein.

Fatty acid analysis

Mid-log phase cultures of *S. bacillaris* NJ-10, *S. minutus* NJ-17 and *S. bacillaris* FACHB753 grown at 4, 10, 15 and 25°C were harvested and freeze-dried. 0.3 g of freeze-dried samples were used for the total lipids extraction with the addition of petroleum ether–diethyl ether (1:1, v/v) for 3 h and transesterified in 1 ml of 0.4 M KOH–MeOH for 3 h. Then, ddH_2O was added to the mixture for stratification and the fatty acid methyl esters in the supernatants were analyzed by gas chromatography (HP5890E, Hewlett-Packard, Wilmington, USA), equipped with a glass column (1.8 m × 2 mm) packed with 5% (w/v) DEGS (Diethylene Glycol Succinate) as stationary phase. The flow rate was maintained at 30 ml min^{-1} with N_2 as the carrier gas. The column was held at 180°C and the injector and FID detector were set at 220 and 240°C, respectively. Fatty acids were identified according to retention time comparison with the internal standard.

Results and discussion

Two *Stichococcus* species isolated from Antarctica

Based on the 18S rDNA sequences, two strains NJ-10 and NJ-17 from Antarctica were identified to be *Stichococcus* species. NJ-10 and NJ-17 were most closely related to *S. bacillaris* s3 and *S. mirabilis* CCAP379/3, respectively, with twenty-nine and eight different bases (Fig. 1). Consistent with the previous studies (Handa et al. 2003; Neustupa et al. 2007), different isolates attributed to *S. bacillaris* do not branch together in the phylogenetic tree, which shows a great difference in the 18S rDNA sequences of this species.

NJ-10 cells were cylindrical, 2.3–3.5 μm broad and 1.6–3.0 times as long and formed short filaments composed of 2–6 cells, which very readily broke apart and slightly constricted at cross walls. A lateral and plate-like chloroplast without pyrenoids covered only a small portion of the cell (Fig. 2a). Cells of NJ-17 were small, short cylindrical, with rounded ends, 1.7–2.6 μm broad and 3.5–5.6 μm long, and no elliptic cells were observed (Fig. 2b). This alga forms short filaments with a plate-like chloroplast occupying a slightly terminal lateral portion. Under transmission electron microscopy, cells of the two *Stichococcus* strains exhibited thick colloid out of cell walls and several big lipid droplets within cytoplasm (Fig. 2c, d). In addition, multiple starch grains were observed in the chloroplast of NJ-10 (Fig. 2c). However, Massalski et al. (2001) did not find starch grains in *S. bacillaris*. On the basis of the morphology, NJ-10 and NJ-17 was identified to be *S. bacillaris* and *S. minutus*, respectively (Ettl and Gärtner 1995).

Species of genus *Stichococcus* distribute widely and have a good adaptation to the extremely low temperature (Broady 1996). *S. bacillaris* is a ubiquitous unicellular eukaryotic terrestrial alga found in ice-free areas

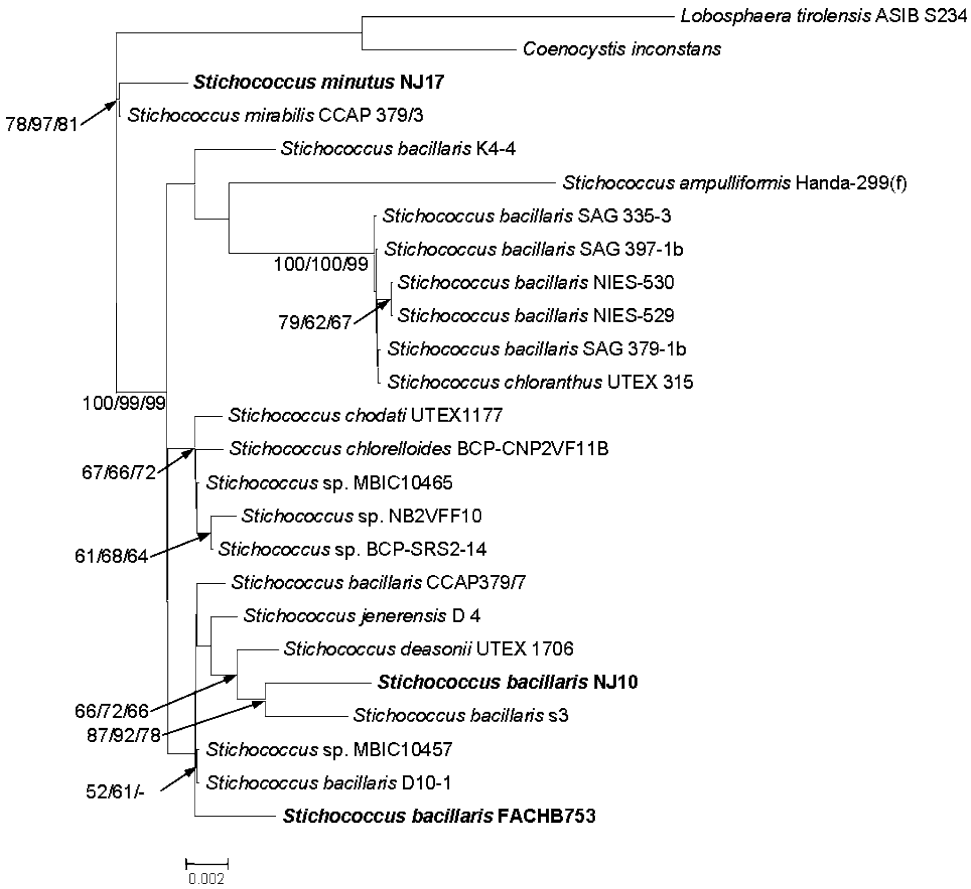

Fig. 1 Phylogenetic consensus tree of *Stichococcus* species as calculated by a PUZZLE analysis inferred from 18S rDNA gene sequences. Bootstrap values are shown at the internal nodes for maximum likelihood (ML; 100 replications), neighbor joining (NJ; 1,000 replications), and maximum parsimony (MP; 1,000 replications), respectively, if the node is supported by at least two bootstrap values of 50% or above. Branch lengths correspond to evolutionary distances. A distance of 0.002 is indicated by the scale

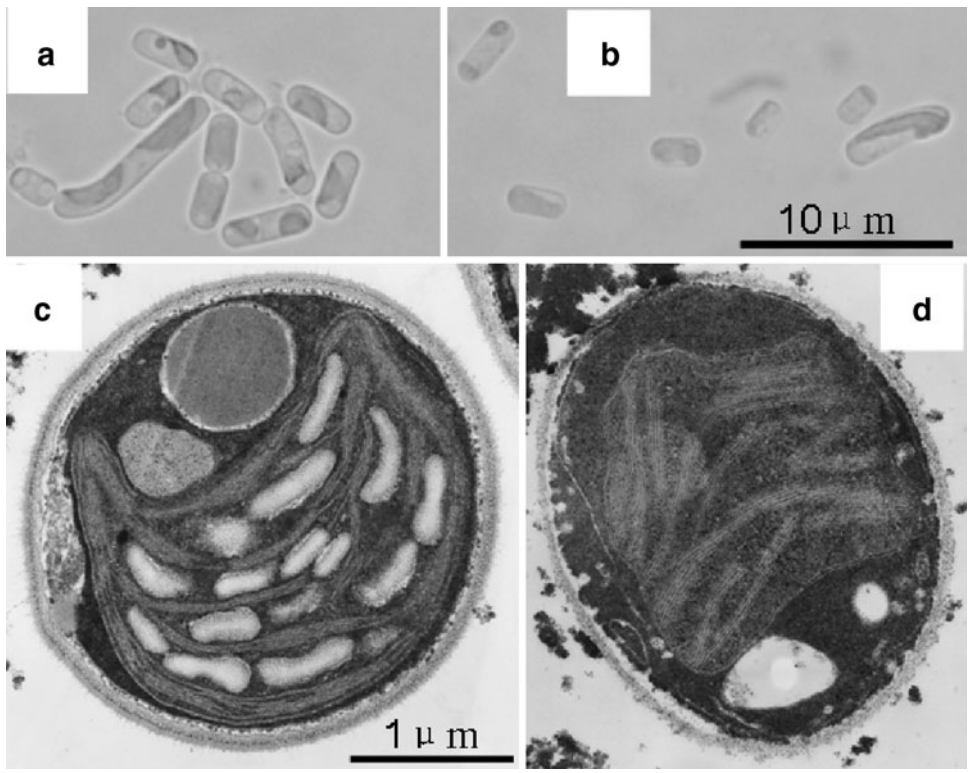

Fig. 2 Morphology of *S. bacillaris* NJ-10 (**a**, **c**) and *S. minutus* NJ-17 (**b**, **d**)

throughout Antarctica (Vinocur and Izaguirre 1994; Broady 1996; Massalski et al. 2001) and its physiological and behavioral adaptations allow it to exist in the most extreme environments (Hughes 2006). In addition, *Stichococcus* is a potential candidate for biodiesel production for its high biomass and lipid content under autotrophic conditions (Olivieri et al. 2011). In this study, big lipid droplets were observed in the cytoplasm of *S. bacillaris* NJ-10 and *S. minutus* NJ-17 together with a good adaptation to the extreme environments, which indicated that the two Antarctic strains might serve as feedstocks for biodiesel production.

Relationship between pre-cultivation temperature and freeze tolerance capacity

Two Antarctic *Stichococcus* strains grew at temperature from 4 to 25°C with higher specific growth rates than the temperate strain *S. bacillaris* FACHB753 (Fig. 3a). *S. bacillaris* FACHB753 could grow above 30°C but could not survive at 4°C, while the two Antarctic strains failed to grow above 25°C. Temperature dependence of the growth of the two Antarctic strains demonstrated that they were psychrotolerants not psychrophiles, consistent with the fact that many microalgae isolated from the polar habitats are psychrotolerants (Teoh et al. 2004).

Freeze tolerance capacity of two Antarctic strains NJ-10 and NJ-17 represented by the ability to reinitiate growth in liquid medium after freezing was shown in Fig. 3b. When pre-cultivated at 4–15°C, strains NJ-10 and NJ-17 exhibited the capacity for freeze tolerance, which did not decrease with the raising of pre-cultivating temperature. Once the pre-cultivating temperature was increased to 25°C, NJ-17 was deprived of the freeze tolerance capacity and NJ-10 could still survive (Fig. 3b). Likewise, in our previous study on the *Chlorella* from Antarctica different freeze tolerance modes were found (Hu et al. 2008).

Photosynthesis, nitrate reductase activity and fatty acid composition

Photosynthesis and respiration rates of the three *Stichococcus* species grown at 10°C were measured from 5 to 35°C (Table 1). The photosynthetic rate of NJ-10, NJ-17 and FACHB753 was 70, 76 and 30 μmol O_2 (mg chl *a* h)$^{-1}$ at 5°C, respectively, and was enhanced with the raising of temperature. The photosynthetic rate in NJ-17 and FACHB753 peaked at 30°C, reaching a value of 198 and 112 μmol O_2 (mg chl *a* h)$^{-1}$, respectively, while in NJ-10 the photosynthetic rate peaked at 25°C with a value of 243 μmol O_2 (mg chl *a* h)$^{-1}$. However, the respiration rate of NJ-17 and FACHB753 reached the maximum at 25°C, and in NJ-10 it peaked at 30°C. In contrast to FACHB753, NJ-10 and NJ-17 shared a high photosynthetic capacity of growth at low temperatures (<10°C). Since growth rate and photosynthetic rate appeared highly correlated in phytoplankton (Coles and Jones 2000), the high photosynthetic activity of NJ-10 and NJ-17 at low temperatures might be conducive to their adaptation to Antarctica.

The activities of nitrate reductase in crude cell extracts of *Stichococcus* were measured from 5 to 40°C and the temperature profile of nitrate reductase activity of the two Antarctic *Stichococcus* was shifted toward lower

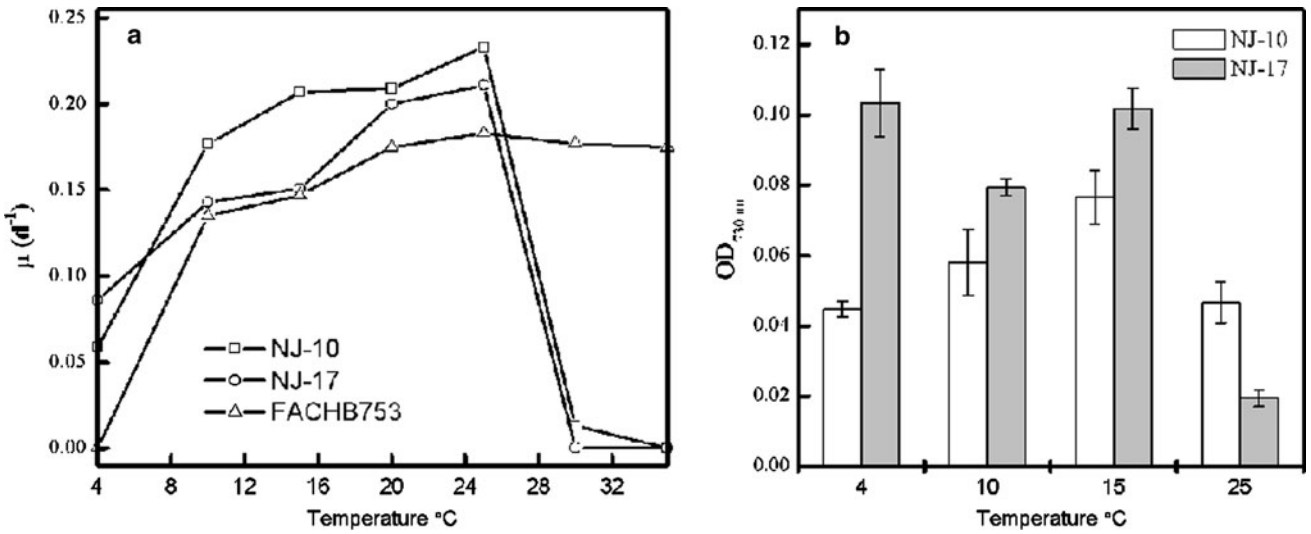

Fig. 3 Temperature dependence of growth (**a**) of *S. bacillaris* NJ-10, *S. minutus* NJ-17 and *S. bacillaris* FACHB753 and the freeze tolerance capacity (**b**) of frozen-thawed cell suspension pre-treated at the indicated temperatures of the two Antarctic *Stichococcus* strains was evaluated by determining the ability of reinitiate of growth (*Y*-axis stands for the turbidity of cells grown for 12 days at 4°C starting from $OD_{730} = 0.04$)

Table 1 Effect of various temperatures on photosynthesis and respiration of *Stichococcus* strains

Organism	*T* (°C)	Photosynthesis [μmol O_2 (mg chl *a* h)$^{-1}$]	Respiration [μmol O_2 (mg chl *a* h)$^{-1}$]
NJ-10	5	70.0 ± 6.0	4.6 ± 0.3
	10	79.1 ± 2.1	14.4 ± 4.1
	15	115.0 ± 8.1	30.2 ± 2.0
	20	129.3 ± 3.1	48.9 ± 2.0
	25	243.2 ± 8.7	56.3 ± 6.5
	30	215.8 ± 0.0	82.1 ± 2.3
	35	36.4 ± 7.0	22.1 ± 9.5
NJ-17	5	75.5 ± 0.0	10.9 ± 0.0
	10	98.5 ± 4.3	14.1 ± 0.0
	15	112.5 ± 0.1	22.5 ± 0.2
	20	113.8 ± 6.6	65.8 ± 6.9
	25	125.8 ± 0.0	83.0 ± 7.6
	30	198.3 ± 0.0	59.2 ± 21.2
	35	103.1 ± 11.5	50.0 ± 5.4
FACHB753	5	29.5 ± 0.0	7.2 ± 0.0
	10	35.3 ± 9.2	12.5 ± 0.0
	15	46.0 ± 1.3	16.5 ± 5.0
	20	56.1 ± 0.0	56.9 ± 1.3
	25	82.6 ± 6.9	65.0 ± 11.7
	30	112.4 ± 0.0	44.2 ± 6.6
	35	70.1 ± 5.8	48.5 ± 14.1

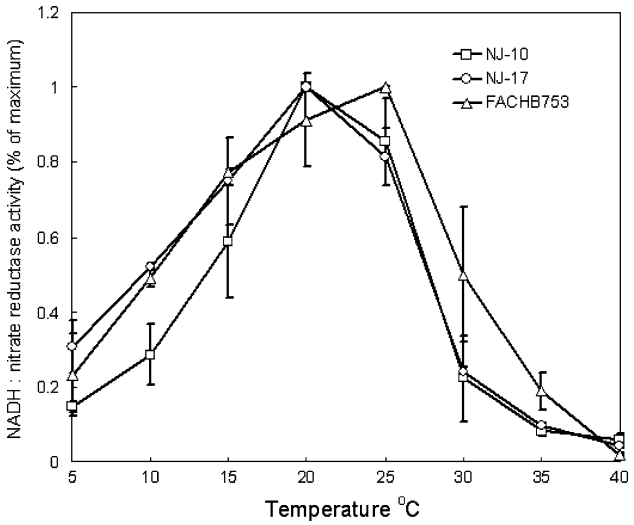

Fig. 4 Temperature dependence of NADH: nitrate reductase activity in cell free extracts of *Stichococcus* strains

temperature compared with that of the temperate *Stichococcus* (Fig. 4). NJ-10 and NJ-17 exhibited the maximum activity at 20°C in vitro, with 15 and 26 nmol NO_2^- min^{-1} mg^{-1} protein, respectively, while the maximum activity of nitrate reductase was found at 25°C with a

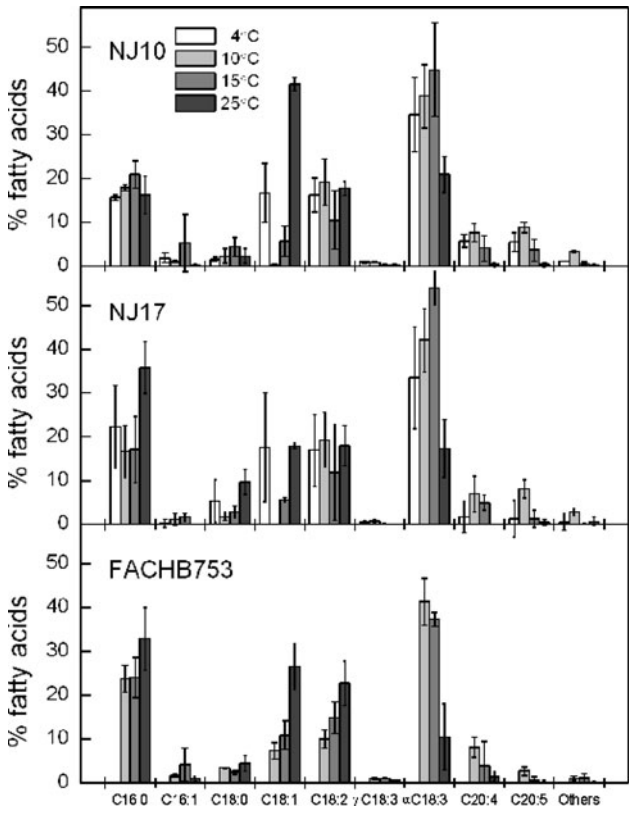

Fig. 5 The fatty acid compositions of *S. bacillaris* NJ-10, *S. minutus* NJ-17 and *S. bacillaris* FACHB753 cultured at 4, 10, 15 and 25°C

value of 13 nmol NO_2^- min^{-1} mg^{-1} protein in FACHB753. Our results indicate that the two Antarctic algae, especially NJ-17, which shows a high level of nitrate reductase specific activity, share same properties which are typical of cold-adapted organisms (Feller and Gerday 1997; Vona et al. 2004; Di Martino Rigano et al. 2006).

The predominant fatty acids were palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (αC18:3) in *Stichococcus* (Fig. 5). The percentage of C16:0, C18:1 and C18:2 in FACHB753 increased at temperatures from 10 to 25°C and that of αC18:3, C20:4 and C20:5 decreased accordingly, in particular, the lowest content of αC18:3 in the three *Stichococcus* strains was achieved at 25°C. Many studies indicated that decreased temperatures would result in a higher proportion of unsaturated or short-chain fatty acids conferring the algae cold tolerance and resistance at low temperatures (Nagashima et al. 1995). In this study, a large portion of unsaturated fatty acids in the two Antarctic species was detected when cultured at 4–15°C. Though many studies reported that decreased temperature would result in increased fatty acid unsaturation (Thompson et al. 1992; Nagashima et al. 1995), the content of αC18:3 in the two Antarctic species increased with the temperatures elevated from 4 to 15°C. It was indicated that the adaptation to cold conditions of these

two Antarctic *Stichococcus* strains was not entirely dependent on the content of unsaturated fatty acids.

The comparison of the different physiological responses to temperature of one temperate and two Antarctic *Stichococcus* strains would improve our understanding of psychrotolerants' adaptive strategies to permanently cold environments. It seems that elevated nitrate reductase activity and photosynthetic rates at low temperatures together with the high proportion of unsaturated fatty acids allow the two *Stichococcus* species to thrive in Antarctica.

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