Identification of a gene, *ccr-1* (*sll1242*), required for chill-light tolerance and growth at 15 °C in *Synechocystis* sp. PCC 6803

Chuntao Yin, Weizhi Li, Ye Du, Renjiu Kong and Xudong Xu

Correspondence

Xudong Xu
xux@ihb.ac.cn

The State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei 430072, China

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Synechocystis sp. PCC 6803 exposed to chill (5 °C)-light (100 µmol photons m⁻² s⁻¹) stress loses its ability to reinitiate growth. From a random insertion mutant library of *Synechocystis* sp. PCC 6803, a *sll1242* mutant showing increased sensitivity to chill plus light was isolated. Mutant reconstruction and complementation with the wild-type gene confirmed the role of *sll1242* in maintaining chill-light tolerance. At 15 °C, the autotrophic and mixotrophic growth of the mutant were both inhibited, paralleled by decreased photosynthetic activity. The expression of *sll1242* was upregulated in *Synechocystis* sp. PCC 6803 after transfer from 30 to 15 °C at a photosynthetic photon flux density of 30 µmol photons m⁻² s⁻¹. *sll1242*, named *ccr* (cyanobacterial cold resistance gene)-1, may be required for cold acclimation of cyanobacteria in light.

INTRODUCTION

Cyanobacteria are a group of oxygenic photosynthetic prokaryotes distributed in a vast array of habitats, including open seas, inland lakes, freshwater ponds of polar regions, hot springs and certain terrestrial environments (Castenholz, 2001). In temperate and subtropical zones, they are adapted to fluctuations of temperature over four seasons, typically from 4 to >30 °C. *Synechocystis* sp. PCC 6803 is a unicellular mesophilic cyanobacterium whose optimal temperature for autotrophic growth is ~30 °C, and preferred temperature between 25 and 40 °C (Tasaka *et al.*, 1996). It is one of the research models for the biology of cyanobacteria, including cold acclimation.

The cold tolerance of cyanobacteria is one of the key factors that limit their global distribution. Genes involved in cold acclimation in cyanobacteria may also be applied to the genetic modification of higher plants. It has been established that the fluidity of the biomembrane plays an important role in the cold acclimation of cyanobacteria and that it is affected by the degree of unsaturation of membrane lipids (Tasaka *et al.*, 1996; Szalontai *et al.*, 2000; Inaba *et al.*, 2003). Four types of acyl-lipid desaturases found in cyanobacteria introduce double bonds at the Δ6, Δ9, Δ12 and ω3 positions of C₁₈ fatty acids, and they are encoded by *desD*, *desC* (*desC1* and *desC2*), *desA* and *desB*, respectively (Murata & Wada, 1995; Chintalapati *et al.*, 2006). When the temperature is lowered, transcripts of *desA*, *desB* and *desC* accumulate in *Synechococcus* sp. PCC 7002, transcripts of *desA*, *desB* and *desD* accumulate in *Synechocystis* sp. PCC 6803, and the level

of unsaturation of membrane glycerolipids is increased (Sakamoto *et al.*, 1997; Kis *et al.*, 1998). Consequently, the fluidity of the membrane is maintained at low temperature (Murata & Wada, 1995; Sakamoto & Murata, 2002). Such a low temperature-induced expression requires light (Kis *et al.*, 1998). In different species, a combination of cold and light stresses may exert different effects on the cell activities of mutants with reduced membrane fluidity. In *Synechocystis* sp. PCC 6803, the decrease of membrane fluidity may depress the recovery of the photosystem II (PSII) protein complex from photoinhibition damage, reducing photosynthesis activity (Gombos *et al.*, 1992; Tasaka *et al.*, 1996). In *Synechococcus* sp. PCC 7002, a high light-tolerant species, however, no such effect has been found (Sakamoto & Bryant, 2002).

Low temperature also affects the expression of other genes. Heat-shock proteins ClpB, ClpP1, HtpG and GroEL are required for cold tolerance and/or are upregulated at low temperature (Porankiewicz & Clarke, 1997; Porankiewicz *et al.*, 1998; Hossain & Nakamoto, 2002). Transcripts and/or proteins encoded by *rbp* (RNA-binding protein) (Sato, 1995), *crhC* (RNA helicase) (Chamot *et al.*, 1999; Chamot & Owttrim, 2000) and *btpA* (required for stabilization of photosystem I, PSI) (Zak & Pakrasi, 2000) accumulate at low temperature in cyanobacteria. With the application of DNA microarray analysis, many more genes have been shown to be up- or down-regulated in the cold (Suzuki *et al.*, 2001).

Random insertion mutagenesis is an alternative method to transcriptomic analysis to find genes involved in cold acclimation. In the study of the chill-light sensitivity of a *desD* mutant of *Synechocystis* sp. PCC 6803, we found a clear

Abbreviations: ARG, ability to reinitiate growth; Em, erythromycin; Km, kanamycin.

difference between this mutant and the wild-type strain. Based on this finding, we attempted to screen chill-light-sensitive mutants from a mutant library of *Synechocystis* sp. PCC 6803 and to identify novel genes involved in cold acclimation. In this paper, we report that *ccr-1* (*sll1242*), whose homologues are widely distributed in mesophilic cyanobacteria, is required for growth at low temperature.

METHODS

Strains, culture conditions and transformation. *Synechocystis* sp. PCC 6803 was from J. Zhao, Peking University, and was cultured in BG11 with or without glucose (5 mM) in 250 to 500 ml flasks at 30 °C under continuous illumination at 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Mutants were grown in the medium with kanamycin (Km, 20 $\mu\text{g ml}^{-1}$) or erythromycin (Em, 5 $\mu\text{g ml}^{-1}$), or both.

Transformation of *Synechocystis* sp. PCC 6803 and mutants was performed according to Williams (1988). The complete segregation of a mutant was confirmed by PCR.

Evaluation of the sensitivity of a strain to cold or chill-light stress. The sensitivity of a strain to 15 °C was calculated as the percentage of its autotrophic growth rate at this temperature compared with that of the wild-type. Strains were inoculated in 500 ml flasks in triplicate with initial OD₇₃₀ 0.2, and grown at 30, 20 or 15 °C on a shaker (120 r.p.m.) at a photosynthetic photon flux density of 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The turbidity was measured every 24 h, and growth rates in the exponential phase were calculated. The tolerance of a strain to 5 °C was calculated as the percentage of its ability to reinitiate growth (ARG) at this temperature compared with that of the wild-type. Cells grown at 30 °C were diluted with BG11 to OD₇₃₀ 0.05 in 100 ml flasks, and 5 ml aliquots were taken from the flasks and transferred into test tubes in triplicate, and exposed to chill stress (5 $\pm$ 1 °C) with or without light (100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 5 days. Sterile 1 M glucose solution (25 μl) was added to each test tube after exposure to the chill stress, and the test tubes were warmed to 30 °C to allow the treated cells to grow at a photosynthetic photon flux density of 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 3.5 days. Samples not exposed to the chill stress were used as the control. The ARG was calculated as OD₇₃₀ (treated)/OD₇₃₀ (control) $\times$ 100 %, in which OD₇₃₀ is the turbidity after photomixotrophic growth at 30 °C for 3.5 days. Data represent the mean $\pm$ SD of values from three tests.

To find the correlation between the ARG and log(c.f.u. ml⁻¹), we also spread the cells on BG11 + glucose plates after the chill stress, employing different 10-fold dilutions to determine the ability of cells to form colonies under photomixotrophic conditions.

Screening of mutants of increased sensitivity to chill-light stress and localization of the antibiotic-resistance marker. A random insertion mutant library of *Synechocystis* sp. PCC 6803 was generated as previously reported (Kong *et al.*, 2003), with modifications. The genomic DNA library constructed with pUC19 was partially digested with *AluI* and *HaeIII*, instead of *Sau3AI*, until most of the plasmid DNA was cut once. DNA fragments larger than 6 kb were eluted from an electrophoresis agarose gel. A Km^r marker was excised from pHB168 (R. Kong & X. Xu, unpublished results) with *KpnI*, blunted with T4 DNA polymerase, ligated with the partially digested library DNA fragments, and electroporated into *Escherichia coli* DH10B, resulting in a random insertion library in *E. coli*. The Km^r marker was derived from C.K2 of pRL446 (Elhai & Wolk, 1988), called C.K2d in this report for convenience. The total plasmid

DNA of the secondary library was then pooled together and used to transform *Synechocystis* sp. PCC 6803. Transformants were maintained in BG11 + glucose with Km in 96-well microtitre plates.

The library was replicated, producing two additional copies; one copy was maintained at 30 °C at a photosynthetic photon flux density of 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and the other was incubated at 5 °C and 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 5 days. Mutants exposed to chill-light stress were allowed to grow at 30 °C for 4 days. Those unable to grow were regarded as candidates for increased chill-light sensitivity and subjected to further screening under the same conditions. Candidate mutants were confirmed by evaluating tolerance to chill-light stress as described above, and those with an ARG < 20 % of that of the wild-type were considered to have significantly increased chill-light sensitivity.

Inserted genes were determined by inverse PCR, sequencing and similarity searches in the Kazusa cyanobacterial genome database (www.kazusa.or.jp/cyano/cyano.html).

PCR and RT-PCR. PCRs for the purpose of molecular cloning were conducted using *Taq/Pfu* (1:1) DNA polymerases (Fermentas UAB). dA was added to the ends of PCR products using *Taq* DNA polymerase before cloning into pMD18-T (Takara), a T vector.

To generate a DNA fragment containing *sll1242::C.K2d*, PCR was performed with the genomic DNA of the *sll1242::C.K2d* mutant as the template, using primers *sll1242-1* (caaagggtgatgtagtctca) and *sll1242-2* (gggcttgggaaactgtcgga).

Inverse PCR was performed with genomic DNA restricted and self-ligated. One microgram of genomic DNA from the mutants was digested with *Sau3AI*, self-ligated in a 50 μl reaction system at 16 °C for 24 h, precipitated with ethanol, and redissolved in 10 μl distilled water. Two microlitres of the self-ligated DNA was used in PCR using primers C.K2-16 (actggcagagcattacgctg)/C.K2-21 (tcaccgaggcagttccatag) or C.K2-8 (cctctccgaccatcaagca)/C.K2-15 (ttgagacacaactggct).

RT-PCR was conducted as previously described (Ning & Xu, 2004). The relative concentration of cDNA templates was evaluated after serial twofold dilutions by PCRs using primers 16SRNA-1 (cctggctcaggatgaacgctg) and 16SRNA-2 (gacgcgccatcttcaga) for the cDNA of 16S rRNA. Primers used for *sll1242* were *sll1242rt-1* (aagttggtcgagtgaggag) and *sll1242rt-2* (tcgccccaaccaactcgt). Two independent experiments were performed, which showed consistent results.

Construction of plasmids

Molecular manipulations were performed by standard protocols. Molecular-tool enzymes were used according to the instructions provided by the manufacturers. Sticky ends of DNA fragments were blunted using T4 DNA polymerase (Promega). PCR products were purified by recovery from agarose gels using a DNA gel extraction kit (Jingmei) and cloned using pMD18-T. Restriction enzymes and T4 DNA ligase were purchased from Takara.

Construction of the plasmid for targeted insertion in *desD*. A DNA fragment containing *desD* generated by PCR using primers *sll0262-1* (caacctttggtcagcatcgg) and *sll0262-2* (tggttccaggcatctgct) was cloned into pMD18-T and inserted at the *BalI* site of *desD* by C.K2 excised from pRL446 with *PvuII*, resulting in pHB451.

Construction of the plasmid for complementation of the *desD::C.K2* mutant. The DNA fragment containing *desD* was generated by PCR using primers *desD-1* (actgctgatcctctggactc) and *desD-2* (ggcaatgtcacgatgctttg), cloned into the *SmaI* site of pUC19, and confirmed by sequencing, resulting in pHB654. C.CE2, a Cm^r and Em^r cassette, was excised from pRL598 (Elhai & Wolk, 1988)

with *Xba*I and inserted into the *Xba*I site in pHB654, resulting in pHB674. The *desD*-C.CE2 fragment was then excised from pHB674 with *Pst*I and *Kpn*I, blunted, and ligated with *Eco*RI-cut and T4 DNA polymerase-blunted pKW1188 (Williams, 1988), replacing its *Km*^r fragment. The resulting plasmid pHB687 was used to transform the *desD*::C.K2 mutant.

Construction of the plasmid for complementation of the *sll1242*::C.K2d mutant. The DNA fragment containing *sll1242* was generated by PCR using primers Gp206-3 (caacagaactgccgcagga) and Gp206-6 (agccatagctatggacaaga), cloned into pMD18-T, and confirmed by sequencing, resulting in pHB916. C.CE2 excised from pRL598 with *Sal*I was inserted into the *Sal*I site of pHB916, resulting in pHB937. The *sll1242*-C.CE2 fragment was then excised from pHB937 with *Sph*I and *Kpn*I, and blunted with T4 DNA polymerase. pKW1188 was cut with *Eco*RI, blunted with T4 DNA polymerase, and ligated with the blunted *sll1242*-C.CE2 fragment. The resulting plasmid pHB945 was used to transform the *sll1242*::C.K2d mutant.

Construction of the plasmid containing *sll1242*::*luxAB*. The *luxAB*- Ω fragment excised from pRL58 (Black & Wolk, 1993) with *Sma*I was ligated with *Apa*I-cut and T4 DNA polymerase-blunted pHB916. The correct orientation was identified by restriction analysis with *Hind*III, resulting in pHB2119.

Luciferase activity. The activity of bacterial luciferase was assayed according to Elhai & Wolk (1990). *Synechocystis* sp. PCC 6803 *sll1242*::*luxAB*- Ω was mixotrophically cultured in 250 ml flasks at 30 °C at a photosynthetic photon flux density of 30 μ mol photons $m^{-2} s^{-1}$ to OD₅₅₀ ~1.0, and transferred to 15 °C at 30 or 3 μ mol photons $m^{-2} s^{-1}$. Samples were taken from the culture at the indicated time intervals and assayed for luciferase activity. Data represent the mean $\pm$ SD of measurements from triplicate samples.

Fatty acid composition. Total lipids of cells grown in BG11 + glucose in 500 ml flasks at 15 °C for 6 days or 30 °C for 4 days (OD₇₃₀ ~1.5) were collected by centrifugation (6000 g), extracted in petroleum ether/diethyl ether (1:1, v/v) for 3 h, and transesterified in 1 ml 0.4 M KOH/methanol. The fatty acid methyl esters were analysed by GC (HP5890E, Hewlett Packard), employing a glass column (1.8 m $\times$ 2 mm) packed with 5% (w/v) diethylene glycol succinate (DEGS). Fatty acids were identified by comparison with the retention times of standards. Quantification was based on known amounts of internal standards (17:0 fatty acid) added to the sample before injection. The relative content of fatty acids (mol%) was expressed with respect to total fatty acids, and data represent the average or mean of values from two or three independent experiments.

Photosynthetic activity. Cells grown autotrophically in 250 ml flasks at 30 °C to OD₇₃₀ 0.8–1.0 were collected by centrifugation, resuspended in fresh BG11 medium in 250 ml flasks to OD₇₃₀ 0.2, and incubated at 15 °C at a photosynthetic photon flux density of 30 μ mol photons $m^{-2} s^{-1}$. Cells were collected by centrifugation at 15 °C after 1, 2 and 3 days, and resuspended in fresh BG11 medium to OD₇₃₀ 1.0. Oxygen evolution rates were measured with a Clark-type oxygen electrode at 15 °C with a saturating actinic light of 2000 μ mol photons $m^{-2} s^{-1}$. Data represent the mean $\pm$ SD of measurements from three independent assays.

RESULTS AND DISCUSSION

Selection of mutants with increased sensitivity to chill-light stress

Synechocystis sp. PCC 6803 kept at 5 °C without light could survive for >2 months (data not shown). However, if

Fig. 1. Loss of c.f.u. of *Synechocystis* sp. PCC 6803 exposed to light at 5 °C. The decrease of ARG after chill-light stress (data not shown) was consistent with that of log₁₀(c.f.u. ml⁻¹). ◇, light at 100 μ mol photons $m^{-2} s^{-1}$; ■, dark.

exposed to light of 100 μ mol photons $m^{-2} s^{-1}$, cells completely lost viability, as seen from the c.f.u. ml⁻¹ (Fig. 1) and ARG results (data not shown), at 5 °C within 10 days. To set up the conditions to screen for mutants of increased chill-light sensitivity, we first tested a *desD*::C.K2 mutant (Fig. 2A) of *Synechocystis* sp. PCC 6803. After exposure to the chill-light stress for 5 days, the ARG of the *desD*::C.K2 mutant decreased to 5.6 ± 3.0 % of that of the wild-type strain (Table 1). In this study, the percentage ARG relative to the wild-type was used to evaluate the tolerance of a mutant to chill-light stress. Complementation of the mutant by integration of *desD* (Fig. 2A) into a platform (Williams, 1988) in the genome restored the ARG to 113.6 ± 52.0 %. The increased sensitivity of the *desD* mutant to the chill-light stress probably reflects the role of 18:3 fatty

Fig. 2. Maps of *desD* (A) and *sll1242* (B) regions showing the locations of C.K2 and C.K2d in different strains. The complement fragments were integrated into the genome via a platform carried on pKW1188 (Williams, 1988).

Table 1. Sensitivity of the *sll1242::C.K2d* mutant to chill-light stress

Values shown are the ratio (%) of the ARG of the mutant to that of the wild-type. All determinations were carried out at 5 °C. ND, Not determined.

Strains	Light intensity (μmol photons m ⁻² s ⁻¹)		
	100	15	0
<i>desD::C.K2</i>	5.6 ± 3.0	5.8 ± 3.7	63.5 ± 9.5
Complemented <i>desD::C.K2</i>	113.6 ± 52.0	ND	ND
<i>sll1242::C.K2d</i>	4.4 ± 1.8	23.6 ± 4.5	73.8 ± 6.2
Complemented <i>sll1242::C.K2d</i>	93.2 ± 13.0	ND	ND
<i>sll0726::C.K2d</i>	4.0 ± 0.4	47.2 ± 3.5	80.7 ± 19.7
<i>sll0158::C.K2d</i>	5.5 ± 4.5	93.1 ± 10.4	92.9 ± 18.5

acids in chill-light tolerance. On the other hand, these results showed the efficacy of chill plus light in identifying a candidate gene required for cold acclimation.

Based on a previously reported procedure (Kong *et al.*, 2003), but taking measures to avoid insertion by multiple DNA fragments, we constructed a random insertion mutant library of *Synechocystis* sp. PCC 6803. From about 6000 Km-resistant colonies, we identified 14 mutants of increased chill-light sensitivity whose ARG under chill-light conditions was <20 % of that of the wild-type. As shown by inverse PCR and sequencing, six interrupted genes were identified in these mutants, including *sll0158* (*glgB*), *sll0726* (*pgm*), *sll0268*, *sll1242*, *sll1913* and *slr0688*.

***sll1242* is required for chill-light tolerance**

The sensitivity of the selected mutants to chill-light stress could be caused by chill or light, or both. We tested these mutants at 5 °C exposed to different light intensities. In the dark, all mutants showed high ARG values; exposed to light of 100 μmol photons m⁻² s⁻¹, all of them showed significantly increased sensitivity (ARG <10 % of that of the wild-type) (Table 1). However, at 15 μmol photons m⁻² s⁻¹, the mutant *sll1242::C.K2d* showed an ARG higher than that of *desD::C.K2*, but significantly lower than that of the other two mutants, *sll0158::C.K2d* and *sll0726::C.K2d* (Table 1). Therefore, *sll1242::C.K2d*, like *desD::C.K2*, was more sensitive to low light at 5 °C than the other two mutants. At 30 °C, these two mutants grew as the wild-type, even at 100 μmol photons m⁻² s⁻¹ (data not shown). Apparently, it was the combination of chill and light that impaired mutants *sll1242::C.K2d* and *desD::C.K2*, with the light playing a crucial role.

The C.K2d Km-resistance fragment was inserted at the *AluI* site 65 bp from the 5' end of *sll1242* (Fig. 2B). To confirm the role of *sll1242* in chill-light tolerance, we generated a PCR product containing *sll1242::C.K2d* and transformed *Synechocystis* sp. PCC 6803 with the DNA fragment to reconstruct the *sll1242* mutant. The phenotype of the reconstructed mutant was identical to that of the original (data not shown). In addition, complementation of the

mutant with *sll1242* restored its tolerance to chill-light stress (Fig. 2B, Table 1).

***sll1242* is required for autotrophic growth at 15 °C**

At a photosynthetic photon flux density of 30 μmol photons m⁻² s⁻¹, *Synechocystis* sp. PCC 6803 grows autotrophically at 15 °C with 0.47 ± 0.03 doublings per day. Under the same conditions, the *sll1242::C.K2d* mutant grew at 0.16 ± 0.02 doublings per day, 34.5 % that of the wild-type. When complemented by wild-type *sll1242*, the percentage increased to 85.5 %. At 30 °C, the mutant (0.92 ± 0.05 doublings per day) was similar to the wild-type (1.03 ± 0.08 doublings per day). As described elsewhere (Sakamoto & Bryant, 1998), *Synechocystis* sp. PCC 6803 cells aggregate at 15 °C. We examined the wild-type and mutant cells under the microscope and found that both formed aggregates of comparable size (data not shown).

We compared the photosynthesis activity of the *sll1242* mutant with that of the wild-type after incubation at 15 °C at a photosynthetic photon flux density of 30 μmol photons m⁻² s⁻¹. Before the treatment, photosynthetic activities were about the same in the mutant and wild-type. After incubation at 15 °C for over 24 h, the mutant gradually lost photosynthetic activity, while the wild-type showed no reduction (Fig. 3). On the third day at 15 °C, the oxygen evolution rate of the mutant was 24.2 % that of the wild-type [160.6 ± 21.0 μmol O₂ (mg chlorophyll)⁻¹ h⁻¹]. This shows that *sll1242*, at least, is required to maintain the photosynthesis activity at such a low temperature in *Synechocystis* sp. PCC6803. Due to the effect of *sll1242* on chill-light tolerance and autotrophic growth at 15 °C, we named this gene *ccr* (*c*yanobacterial *c*old *r*esistance gene)-1.

In the literature on cold acclimation in *Synechocystis* sp. PCC 6803, a temperature of ~20 °C is usually used for cold stress. However, unlike results with a *desA desD* double mutant (Tasaka *et al.*, 1996), we found much less or no difference between the *sll1242* mutant (0.50 ± 0.03 doublings per day) and the wild-type (0.54 ± 0.01 doublings per day) at 20 °C. The requirement for *sll1242* for growth at 15 °C, but not

Fig. 3. Photosynthesis activities of the wild-type (◆) and *sll1242* mutant (□) incubated in BG11 at 15 °C at a photosynthetic photon flux density of 30 μmol photons m⁻² s⁻¹ for different periods of time. Chl, chlorophyll.

20 °C, suggests that the role of this gene in acclimation to low-temperature stress is different from those of other genes involved in cold acclimation. It is possible that *sll1242* plays a role in cold acclimation only under the harsher stress of low temperature in the presence of light.

Effects of *sll1242* on mixotrophic growth at 15 °C and transcriptional analyses

Under mixotrophic conditions, the growth rate of the *sll1242* mutant was 59.9 % that of the wild-type (0.68 ± 0.09 doublings per day) at 15 °C, and 91.7 % that of the wild-type (1.65 ± 0.04 doublings per day) at 30 °C. At 15 °C, the photosynthetic activity of the mutant was reduced to 40.7 % that of the wild-type [212.4 ± 37.1 μmol O₂ (mg chlorophyll)⁻¹ h⁻¹] within 3 days, which partially explained the inhibition of the mixotrophic growth of the mutant at this temperature.

We employed a DNA microarray to analyse the effect of *sll1242* on gene expression at 15 °C, but found no significant difference between the mutant and wild-type (data not shown). In addition, we analysed the fatty acid composition

of total glycerolipids. As shown in Table 2, in wild-type cells transferred from 30 to 15 °C, 18:3 (9,12,15) increased from 1.35 to 9.46 % and 18:4 (6,9,12,15) from 1.07 to 5.95 %. Fatty acids at 30 °C were essentially unchanged in the mutant, but at 15 °C, 18:3 (9,12,15) and 18:4 (6,9,12,15) were reduced to 5.85 and 3.00 %, respectively, while 18:3 (6,9,12) was not decreased as in the wild-type. Hence, the regulation of fatty acid composition at 15 °C was partially suppressed in the *sll1242* mutant.

Using RT-PCR, we found upregulation of *sll1242* at low temperature (Fig. 4A). Employing the bacterial luciferase reporter gene *luxAB*, we followed the transcription of *sll1242* under different conditions (Fig. 4B, C). The promoter activity of *sll1242* was upregulated after transfer from 30 to 15 °C if the light intensity was set at 30 μmol photons m⁻² s⁻¹, but not at 3 μmol photons m⁻² s⁻¹. Transfer from 30 to 30 °C did not affect the expression. Complementation of the *sll1242::luxAB-Ω* strain indicated that the regulation of its promoter is not self-regulatory. Under mixotrophic conditions, *desA*, *desB* and *desD* genes are upregulated within 1 h upon exposure to low-temperature stress in the presence of light (Kis *et al.*, 1998). However, *sll1242* was not significantly upregulated until 2 days had elapsed. The timing of the regulation of *sll1242* by low temperature is consistent with the fact that it exerts no effect on the expression of *des* and other genes involved in cold acclimation. The effect of light intensity on the upregulation of *sll1242* at 15 °C may be related to its role in maintaining the photosynthesis activity at this temperature.

A BLAST search of conserved domains predicts that *sll1242* may be a Fe-S oxidoreductase family protein (COG1032) and may possess a vitamin B₁₂-binding domain. It remains possible that *sll1242* affects photosynthesis activity by directly or indirectly affecting certain metabolic pathway(s). Its homologues are found in almost all cyanobacteria and many other bacteria (data not shown). Based on the results presented in this paper, we propose that *sll1242*, and possibly its homologues in other cyanobacteria, is required for chill-light tolerance and growth at low temperature.

Table 2. Fatty acid composition (mol%) of the total glycerolipids from the wild-type and *sll1242* mutant strains

Fatty acid	Wild-type		<i>sll1242::C.K2d</i>	
	15 °C	30 °C	15 °C	30 °C
16:0	43.73 ± 3.36	48.67 ± 1.42	48.55 ± 0.85	52.08 ± 2.57
16:1 (9)	7.50 ± 1.21	5.33 ± 1.24	6.84 ± 0.62	4.62 ± 1.57
18:0	1.90 ± 0.04	1.71 ± 0.69	1.96 ± 0.17	1.68 ± 0.60
18:1 (9)	11.42 ± 5.48	12.23 ± 4.53	9.18 ± 2.18	11.59 ± 4.11
18:2 (9,12)	9.67 ± 0.33	15.71 ± 0.93	10.43 ± 0.07	14.02 ± 2.1
18:3 (6,9,12)	10.63 ± 1.50	13.93 ± 3.62	14.52 ± 0.60	13.88 ± 1.67
18:3 (9,12,15)	9.46 ± 1.12	1.35 ± 0.15	5.85 ± 0.31	1.38 ± 0.38
18:4 (6,9,12,15)	5.95 ± 0.69	1.07 ± 0.24	3.00 ± 0.36	0.78 ± 0.34

Fig. 4. Induced expression of *sll1242* in *Synechocystis* sp. PCC 6803 under mixotrophic conditions after transfer from 30 to 15 °C. (A) RT-PCR detection of the relative mRNA level of *sll1242* in cells at 30 °C (4 days; lanes 1–6, serial twofold dilutions of cDNA) and 15 °C (4 days; lanes 7–12, serial twofold dilutions of cDNA) at a photosynthetic photon flux density of 30 μmol photons m⁻² s⁻¹. (B) Insertion of *luxAB* into *sll1242* and complementation. (C) Expression of *luxAB* from the promoter of *sll1242*. ◆, *sll1242::luxAB-Ω* transferred from 30 °C, 30 μmol photons m⁻² s⁻¹, to 15 °C, 30 μmol photons m⁻² s⁻¹; ▲, *sll1242::luxAB-Ω* complemented with wild-type *sll1242* transferred from 30 °C, 30 μmol photons m⁻² s⁻¹, to 15 °C, 30 μmol photons m⁻² s⁻¹; □, *sll1242::luxAB-Ω* transferred from 30 °C, 30 μmol photons m⁻² s⁻¹, to 15 °C, 3 μmol photons m⁻² s⁻¹; ×, *sll1242::luxAB-Ω* transferred from 30 °C, 30 μmol photons m⁻² s⁻¹, to 30 °C, 30 μmol photons m⁻² s⁻¹. Chla, chlorophyll a.

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REFERENCES

- Black, T. A. & Wolk, C. P. (1993). Spatial expression and autoregulation of *hetR*, a gene involved in the control of heterocyst development in *Anabaena*. *Mol Microbiol* **9**, 77–84.
- Castenholz, R. W. (2001). Oxygenic photosynthetic bacteria. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 1,

pp. 474–599. Edited by D. R. Boone & R. W. Castenholz. New York, Berlin, Heidelberg: Springer.

Chamot, D. & Owttrim, G. W. (2000). Regulation of cold shock-induced RNA helicase gene expression in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J Bacteriol* **182**, 1251–1256.

Chamot, D., Magee, W. C., Yu, E. & Owttrim, G. W. (1999). A cold shock-induced cyanobacterial RNA helicase. *J Bacteriol* **181**, 1728–1732.

Chintalapati, S. K., Prakash, J. S. S., Gupta, P., Ohtani, S., Suzuki, I., Sakamoto, T., Murata, N. & Shivaji, S. (2006). A novel Delta9 acyl-lipid desaturase, DesC2, from cyanobacteria acts on fatty acids esterified to the *sn*-2 position of glycerolipids. *Biochem J* **398**, 207–214.

Elhai, J. & Wolk, C. P. (1988). A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers. *Gene* **68**, 119–138.

Elhai, J. & Wolk, C. P. (1990). Developmental regulation and spatial pattern of expression of the structural genes for nitrogenase in the cyanobacterium *Anabaena*. *EMBO J* **9**, 3379–3388.

Gombos, Z., Wada, H. & Murata, N. (1992). Unsaturation of fatty acids in membrane lipids enhances tolerance of the cyanobacterium *Synechocystis* PCC 6803 to low-temperature photoinhibition. *Proc Natl Acad Sci U S A* **89**, 9959–9963.

Hossain, M. M. & Nakamoto, H. (2002). HtpG plays a role in cold acclimation in cyanobacteria. *Curr Microbiol* **44**, 291–296.

Inaba, M., Suzuki, I., Szalontai, B., Kanesaki, Y., Los, D. A., Hayashi, H. & Murata, N. (2003). Gene-engineered rigidification of membrane lipids enhances the cold inducibility of gene expression in *Synechocystis*. *J Biol Chem* **278**, 12191–12198.

Kis, M., Zsiros, O., Farkas, T., Wada, H., Nagy, F. & Gombos, Z. (1998). Light-induced expression of fatty acid desaturase genes. *Proc Natl Acad Sci U S A* **95**, 4209–4214.

Kong, R., Xu, X. & Hu, Z. (2003). A TPR-family membrane protein gene is required for light-activated heterotrophic growth of the cyanobacterium *Synechocystis* sp. PCC 6803. *FEMS Microbiol Lett* **219**, 75–79.

Murata, N. & Wada, H. (1995). Acyl-lipid desaturases and their importance in the tolerance and acclimation to cold of cyanobacteria. *Biochem J* **308**, 1–8.

Ning, D. & Xu, X. (2004). *alr0117*, a two-component histidine kinase gene, is involved in heterocyst development in *Anabaena* sp. PCC 7120. *Microbiology* **150**, 447–453.

Porankiewicz, J. & Clarke, A. K. (1997). Induction of the heat shock protein ClpB affects cold acclimation in the cyanobacterium *Synechococcus* sp. strain PCC 7942. *J Bacteriol* **179**, 5111–5117.

Porankiewicz, J., Schelin, J. & Clarke, A. K. (1998). The ATP-dependent Clp protease is essential for acclimation to UV-B and low temperature in the cyanobacterium *Synechococcus*. *Mol Microbiol* **29**, 275–283.

Sakamoto, T. & Bryant, D. A. (1998). Growth at low temperature causes nitrogen limitation in the cyanobacterium *Synechococcus* sp. PCC 7002. *Arch Microbiol* **169**, 10–19.

Sakamoto, T. & Bryant, D. A. (2002). Synergistic effect of high-light and low temperature on cell growth of the Delta 12 fatty acid desaturase mutant in *Synechococcus* sp. PCC7002. *Photosynth Res* **72**, 231–242.

Sakamoto, T. & Murata, N. (2002). Regulation of desaturation of fatty acids and its role in tolerance to cold and salt stress. *Curr Opin Microbiol* **5**, 208–210.

Sakamoto, T., Higashi, S., Wada, H., Murata, N. & Bryant, D. A. (1997). Low-temperature-induced desaturation of fatty acids and

expression of desaturase genes in the cyanobacterium *Synechococcus* sp. PCC 7002. *FEMS Microbiol Lett* **152**, 313–320.

Sato, N. (1995). A family of cold-regulated RNA-binding protein genes in cyanobacterium *Anabaena variabilis* M3. *Nucleic Acids Res* **23**, 2161–2167.

Suzuki, I., Kanesaki, Y., Mikami, K., Kanehisa, M. & Murata, N. (2001). Cold-regulated genes under control of the cold sensor Hik33 in *Synechocystis*. *Mol Microbiol* **40**, 235–244.

Szalontai, B., Nishiyama, Y., Gombos, Z. & Murata, N. (2000). Membrane dynamics as seen by Fourier transform infrared spectroscopy in a cyanobacterium, *Synechocystis* PCC 6803. The effects of lipid unsaturation and the protein-to-lipid ratio. *Biochim Biophys Acta* **1509**, 409–419.

Tasaka, Y., Gombos, Z., Nishiyama, Y., Mohanty, P., Ohba, T., Okhi, K. & Murata, N. (1996). Targeted mutagenesis of acyl-lipid desaturases in *Synechocystis*: evidence for the important roles of polyunsaturated membrane lipids in growth, respiration and photosynthesis. *EMBO J* **15**, 6416–6425.

Williams, J. G. K. (1988). Construction of specific mutations in photosystem II photosynthetic reaction center by engineering methods in *Synechocystis* 6803. *Methods Enzymol* **167**, 766–778.

Zak, E. & Pakrasi, H. B. (2000). The BtpA protein stabilizes the reaction center proteins of photosystem I in the cyanobacterium *Synechocystis* sp. PCC 6803 at low temperature. *Plant Physiol* **123**, 215–222.

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