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Toshio Sakamoto · Gaozhong Shen · Shoichi Higashi
Norio Murata · Donald A. Bryant

Alteration of low-temperature susceptibility of the cyanobacterium *Synechococcus* sp. PCC 7002 by genetic manipulation of membrane lipid unsaturation

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Abstract Cyanobacteria acclimate to low temperature by desaturating their membrane lipids. Mutant strains of *Synechococcus* sp. PCC 7002 containing insertionally inactivated *desA* (Δ12 acyl-lipid desaturase) and *desB* (ω3 acyl-lipid desaturase) genes were produced, and their low-temperature susceptibility was characterized. The *desA* mutant synthesized no linoleic acid or α-linolenic acid, and the *desB* mutant did not produce α-linolenic acid. The *desA* mutant grew more slowly than the wild-type at 22° C and could not grow at 15° C. The *desB* mutant could not continuously grow at 15° C, although no observable phenotype appeared at higher temperatures. It has been shown that expression of the *desA* gene occurs at 38° C and is up-regulated at 22° C, and that the *desB* gene is only expressed at 22° C. These results indicate that the expression of the *desA* and *desB* genes occurs at higher temperatures than those at which a significant decline in physiological activities is caused by the absence of their products. The temperature dependency of photosynthesis was not affected by these mutations. Since chlorosis and inability to grow at 15° C with nitrate was suppressed by the substitution of urea as a nitrogen source, it is very likely that the chilling susceptibility of the desaturase mutants is attributable to nutrient limitation.

Key words Chilling tolerance · Cyanobacterium · Fatty acid desaturase · Low-temperature acclimation · Membrane lipid · Photosynthesis

T. Sakamoto · G. Shen · D. A. Bryant (✉)
Department of Biochemistry and Molecular Biology,
S-234 Frear Bldg., The Pennsylvania State University,
University Park, PA 16802, USA
Tel. +1 814-865-1992; Fax +1 814-863-7024
e-mail: dab14@psu.edu

S. Higashi and N. Murata
Department of Regulation Biology,
National Institute for Basic Biology, Myodaiji,
Okazaki, 444 Japan
Tel. +81 564-55-7600; Fax +81 564-54-4866
e-mail: murata@nibb.ac.jp

Abbreviations *X:Y(Z)* Fatty acids containing X carbon atoms with Y double bonds at the position Z counted from the carboxyl terminus · *16:0* Palmitic acid · *16:1(9)* Palmitoleic acid · *18:0* Stearic acid · *18:1(9)* Oleic acid · *18:2(9,12)* Linoleic acid · *18:3(9,12,15)* α-Linolenic acid · Km^R Kanamycin resistance gene · *PCC* Pasteur Culture Collection

Introduction

Most organisms, including microorganisms and plants, alter the composition of their membrane lipids to compensate for the decrease of fluidity of the lipid bilayer at low temperature (Russell 1984; Harwood et al. 1994). This adaptive response, known as homeoviscous adaptation of biological membranes (Cossins 1994), has been extensively studied in cyanobacteria by Murata and coworkers [see Murata and Wada (1995) for a review]. However, the role(s) of membrane lipid unsaturation on the low-temperature adaptation and/or acclimation of biochemical reactions is still not well understood.

Cyanobacteria have been classified into four groups based upon their fatty acid compositions and the patterns of desaturation that these exhibit (Murata and Wada 1995). The unicellular marine cyanobacterium *Synechococcus* sp. PCC 7002 is classified as a member of Group 2 (Murata et al. 1992; Sakamoto et al. 1997). *Synechococcus* sp. PCC 7002 synthesizes lipids containing C_{18} fatty acids with none, one, two, or three double bonds at the Δ9, Δ12, and ω3 (or Δ15) positions at the *sn*–1 position, and C_{16} fatty acids containing none or one double bond at the Δ9 position at the *sn*–2 position. Double bonds in the *sn*–1 fatty acid are added sequentially starting with desaturation at the Δ9 position and proceeding to the ω3 position. Three acyl-lipid desaturases, DesC (Δ9 desaturase), DesA (Δ12 desaturase) and DesB (ω3 or Δ15 desaturase), are responsible for the conversion of stearic acid to α-linolenic acid. These three desaturase genes have been cloned, and the expression patterns of three desaturase genes in response to temperature have been characterized in *Syn-*

echococcus sp. PCC 7002 (Sakamoto et al. 1994a; Sakamoto and Bryant 1997a). The *desA* (Δ12 desaturase) and *desC* (Δ9 desaturase) genes were expressed at both 22 and at 38° C, but transcripts of the *desB* (ω3 desaturase) gene are detected only in cells grown at 22° C (Sakamoto and Bryant 1997a; Sakamoto et al. 1997). Consequently, desaturation at the Δ9 and Δ12 positions occurs in cells grown from 22 to 38° C, but desaturation at the ω3 (or Δ15) position occurs only in cells grown at low temperature.

Studies performed by gain-of-function of the *desA* (Δ12 acyl-lipid desaturase) gene demonstrate that unsaturation at the Δ12 position of membrane lipids enhances the low-temperature tolerance of the cyanobacterium *Synechococcus* sp. PCC 7942 under dark conditions (Wada et al. 1990, 1994; H. Wada, Kyushu University, Fukuoka, Japan, personal communication). Loss-of-function analysis of the *desA* gene in *Synechocystis* sp. PCC 6803 shows that polyunsaturated fatty acids are necessary for cell growth and tolerance of photosynthetic activity under excess light conditions at low temperature (Wada et al. 1992). Recent studies have shown that the ability to recover photosynthetic activity after photoinhibitory damage by excess light at low temperature is reduced when the desaturation level of membrane lipids is decreased by genetic manipulation in *Synechocystis* sp. PCC 6803 (Gombos et al. 1992, 1994; Tasaka et al. 1996).

The expression of the *desB* (ω3 or Δ15 desaturase) gene is specifically induced by low temperature (Sakamoto et al. 1994b, 1997), and the greatly increased levels of 18:3 (9,12,15) fatty acids in low-temperature-grown cells is one of the most remarkable phenomena associated with temperature-induced changes of fatty acid composition in cyanobacteria (Sato and Murata 1980; Wada and Murata 1990; Sakamoto et al. 1997). However, no specific role for ω3 desaturation in low-temperature acclimation of cyanobacteria has yet been established. Tasaka et al. (1996) have recently reported that ω3 desaturation has no effect on the low-temperature tolerance of *Synechocystis* sp. PCC 6803.

In the work presented here, *desA* and *desB* mutant strains of *Synechococcus* sp. PCC 7002 were produced by interposon mutagenesis, and growth responses of these desaturase mutants to shifts in temperature were characterized. Our results demonstrate that membrane lipid desaturation at the ω3 position is required for continuous growth at temperatures that are close to the lower limit of growth temperature for this cyanobacterium.

Materials and methods

Organisms, culture conditions, and measurement of growth

Synechococcus sp. PCC 7002 strain PR6000 was obtained and grown in media A^+ or A-U as described (Sakamoto and Bryant 1997b). The growth temperature and light intensity for all experiments are specified in the text, figure legends, and table footnotes. For the selection of kanamycin-resistant mutants, kanamycin (50 μg ml^{-1}) was added to the medium. Growth was monitored by changes in the optical density at 550 nm; the correlation between optical density, microscopic cell count, and viable cell count has been described (Sakamoto and Bryant 1997b). All growth experiments were repeated at least twice.

Interposon mutagenesis of the *desA* and *desB* genes of *Synechococcus* sp. PCC 7002

The *desA* (Δ12 desaturase) gene was originally isolated from a genomic library of *Synechococcus* sp. PCC 7002 (strain NIBB) (Sakamoto et al. 1994a), and the *desB* (ω3 desaturase) gene was isolated from *Synechococcus* sp. PCC 7002 (strain PR6000) (Sakamoto and Bryant 1997a). The nucleotide sequences of these genes are deposited in the GenBank/ DDBJ/ EMBL databases with the following accession numbers: *desA* (D13779) and *desB* (U36389). The *desA* and *desB* genes were inactivated by inserting a DNA fragment (that encodes an aminoglycoside 3′-phosphotransferase and thereby confers resistance to kanamycin) into unique restriction sites within the coding regions of the two genes. To inactivate the *desA* gene, a 3.8-kb *Cla*I-*Pst*I fragment encoding the *desA* gene (Sakamoto et al. 1994a; Sakamoto and Bryant 1997a) was cloned into the *Cla*I and *Pst*I sites of pBluescript KS(–) (Stratagene, La Jolla, Calif., USA). The resultant plasmid was digested with *Hin*dIII and ligated with a 1.6-kb *Hin*dIII fragment encoding the *aph* gene derived from plasmid pUC4 KIXX (Pharmacia, Uppsala, Sweden). In the resulting construction, the direction of transcription of the *aph* gene was opposite that of the *desA* gene. To inactivate the *desB* gene, plasmid pUC19/3.5 kb (Sakamoto and Bryant 1997a) was digested with *Bgl*II and ligated with a 1.4-kb *Bam*HI fragment encoding the *aphII* gene derived from plasmid pRL170 (Elhai and Wolk 1988). The direction of transcription of the *aphII* gene was opposite that of the *desB* gene.

Wild-type cells of *Synechococcus* sp. PCC 7002 (strain PR6000) were transformed with plasmid DNA essentially as described by Stevens and Porter (1980). Kanamycin-resistant transformants were selected at 37° C on medium A^+ plates supplemented with 50 μg kanamycin ml^{-1}. To verify that the mutant strains were homozygous, total genomic DNA was isolated from cells by phenol-chloroform extraction and subsequent ethanol precipitation, and the DNA was analyzed by polymerase chain reaction (PCR). Genomic DNA isolated from the wild-type strain PR6000 served as the control. Specific primers for the *desA* gene were: forward, 5′-AACCATGGCTTAGTTTA-3′; reverse, 5′-AGGAACTAGAAGGTCTC-3′. Those for the *desB* gene were: forward, 5′-GTGGTAGTGGACATAAA-3′; reverse, 5′-GTGAACTGTACTTGACC-3′. The resultant *desA* and *desB* mutant strains were designated PR6080 and PR6081, respectively.

Biochemical analyses and fluorescence spectroscopy

Fatty acids were converted to methyl-esters, extracted with *n*-hexane, and analyzed by gas-liquid chromatography as previously described by Wada and Murata (1990). Photosynthetic oxygen evolution from whole cells was measured with a Clark-type oxygen electrode with 10 mM $NaHCO_3$ as the final electron acceptor as described by Sakamoto and Bryant (1997b). Fluorescence emission spectra at 77K with 440 nm excitation were measured with an SLM 8000C spectrofluorometer (SLM-Aminco Instruments Inc.; now Spectronic Instruments, Rochester, N.Y., USA). Cells (OD_{730} = 1.0) were suspended in 60% (v/v) glycerol in 25 mM Hepes-NaOH (pH 7.0) and quickly frozen in liquid nitrogen prior to measurement.

Results

Interposon mutagenesis of the *desA* and *desB* genes in *Synechococcus* sp. PCC 7002 (strain PR6000)

PCR analyses were performed to confirm that complete segregation of the *desA* and *desA*::*aph* alleles had occurred in the *desA* mutant strain PR6080. The *desA*-specific PCR primers amplified a 1.6-kb DNA fragment

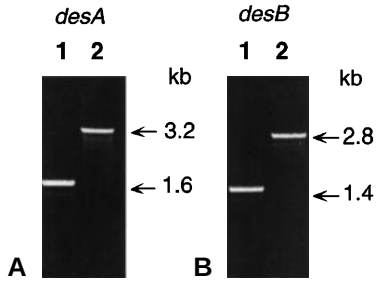


Fig. 1A, B Evaluation of gene replacement in the chromosomal DNAs of the *desA* mutant strain PR6080 and the *desB* mutant strain PR6081 by polymerase chain reaction (PCR) analysis. **A** PCR analysis of the *desA* mutant strain PR6080. Genomic DNA from the wild-type (*lane 1*) and the *desA* mutant (*lane 2*) cells was used as template for PCR with the primers specific for the *desA* gene. **B** PCR analysis of the *desB* mutant strain PR6081. Genomic DNA from the wild-type (*lane 1*) and the *desB* mutant (*lane 2*) cells was used as template for PCR with the primers specific for the *desB* gene. Amplified DNA fragments were separated by agarose gel electrophoresis and detected by ethidium bromide staining

when the template was total genomic DNA of the wild-type strain PR6000 (Fig. 1, panel A, lane 1). When DNA from the *desA* mutant strain PR6080 was used as template, no 1.6-kb product was amplified, but a DNA fragment of 3.2 kb (corresponding to the *desA* gene fragment plus the 1.6-kb *aph* gene insert) was amplified (Fig. 1, panel A, lane 2). These results indicate that the *desA* mutant strain PR6080 is homozygous and contains the expected *aph* insertion in the *desA* gene. Similarly, PCR analyses were used to demonstrate complete segregation of the *desB* and *desB*::*aphII* alleles. When the *desB*-specific PCR primers were used with total genomic DNA from wild-type strain PR6000, a single fragment of 1.4 kb was amplified (Fig. 1, panel B, lane 1). When these primers were used with total DNA extracted from the *desB* mutant strain PR6081, no 1.4-kb product was amplified, but instead a 2.8-kb DNA fragment (corresponding to the *desB* gene fragment with the inserted 1.4-kb *aphII* gene) was amplified (Fig. 1, panel B, lane 2). These results indicate that the *desB* mutant strain PR6081 is homozygous and contains the expected interruption of the *desB* gene.

Fatty acid composition of the desaturase mutants

Table 1 shows the fatty acid composition of total lipids from cells of *Synechococcus* sp. PCC 7002 strains PR6000, PR6080, and PR6081 grown at 38 or 22° C. Wild-type strain PR6000 can synthesize 18:1(9), 18:2(9, 12), and 18:3(9, 12, 15) C_{18} fatty acids. The level of 18:3 (9, 12, 15) fatty acids increased substantially when cells were grown at 22° C. The *desA* mutant strain PR6080 did not contain either 18:2(9, 12) or 18:3(9, 12, 15) fatty acids when grown at either growth temperature, but the level of 18:1(9) fatty acids increased as compared to that of the wild-type strain. A small amount of an unidentified fatty acid was detected in the *desA* mutant PR6080 cells grown at 22° C, and this unknown fatty acid was not observed in cells grown at 38° C. Based on its elution time in the chromatographic system employed, the unknown fatty acid was assumed to be an 18:2 fatty acid [possibly 18:2 (9, 15)], although the actual carbon chain length and the double-bond positions were not determined. These results demonstrate that the *desA* mutant strain PR6080 has lost the ability to synthesize both 18:2(9,12) and 18:3 (9,12,15) fatty acids and that, as expected, the identified *desA* gene encodes the Δ12 acyl-lipid desaturase responsible for the desaturation of fatty acids at the Δ12 position.

The cells of the *desB* mutant strain PR6081 contained 18:1(9) and 18:2(9, 12) but no 18:3(9, 12, 15) fatty acids at either growth temperature tested. When the mutant cells were grown at 22° C, the level of 18:2(9, 12) fatty acid increased, but production of 18:3(9, 12, 15) fatty acid was not detected, as occurs in the wild-type. These results show that the *desB* mutant has lost the ability to introduce a double bond at the Δ15 (or ω3) position at low temperature and that the *desB* gene encodes the Δ15 acyl-lipid desaturase.

Growth of the desaturase mutants in response to temperature

Figure 2 shows the growth profiles of wild-type strain PR6000 cells, *desA* mutant strain PR6080 cells, and *desB* mutant strain PR6081 cells after a temperature shift-down from 38 to 22° C. Wild-type cells of *Synechococcus* sp.

Table 1 Fatty acid composition of total lipids in strains PR6000, PR6080, and PR6081 of *Synechococcus* sp. PCC 7002. The values present the means of at least two experiments. Cells were grown in medium A^+ and 250 μE m^{-2} s^{-1} on 1% CO_2 in air at 38 or 22° C

	Fatty acids (mol%)						
	16:0	16:11 (9)	18:0	18:1 (9)	18:2 (9, 12)	UK[a]	18:3 (9, 12, 15)
Grown at 38° C:							
PR6000 (WT)	55 ± 2	6 ± 1	1	17 ± 3	18 ± 2	0	1
PR6080 (*desA*)	55 ± 1	5 ± 1	1	39 ± 1	0	0	0
PR6081 (*desB*)	59 ± 1	6 ± 1	1	14 ± 1	20 ± 1	0	0
Grown at 22° C:							
PR6000 (WT)	51 ± 2	7 ± 1	1	7 ± 1	15 ± 1	0	19 ± 2
PR6080 (*desA*)	49 ± 1	6 ± 1	1	43 ± 1	0	1	0
PR6081 (*desB*)	49 ± 1	8 ± 1	1	8 ± 1	34 ± 1	0	0

[a] Unknown fatty acid

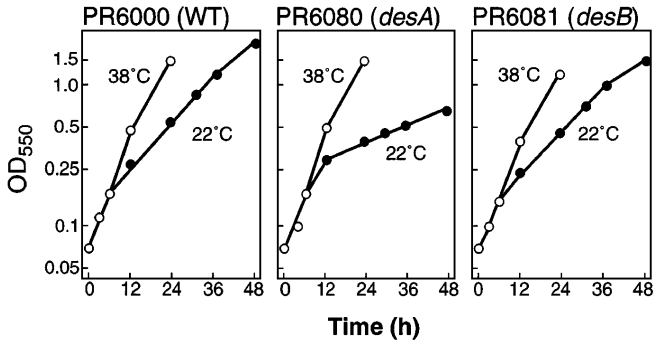


Fig. 2 Growth curves for wild-type strain PR6000, *desA* mutant strain PR6080, and *desB* mutant strain PR6081 at 38° C and after temperature shift-down to 22° C. Cells were grown at 38° C in medium A^+ containing nitrate as a nitrogen source under 250 μE m^{-2} s^{-1} with aeration of 1% (v/v) CO_2 in air (○). After growth for 6 h at 38° C, the culture was divided and a portion was shifted down to 22° C under otherwise identical growth conditions (●)

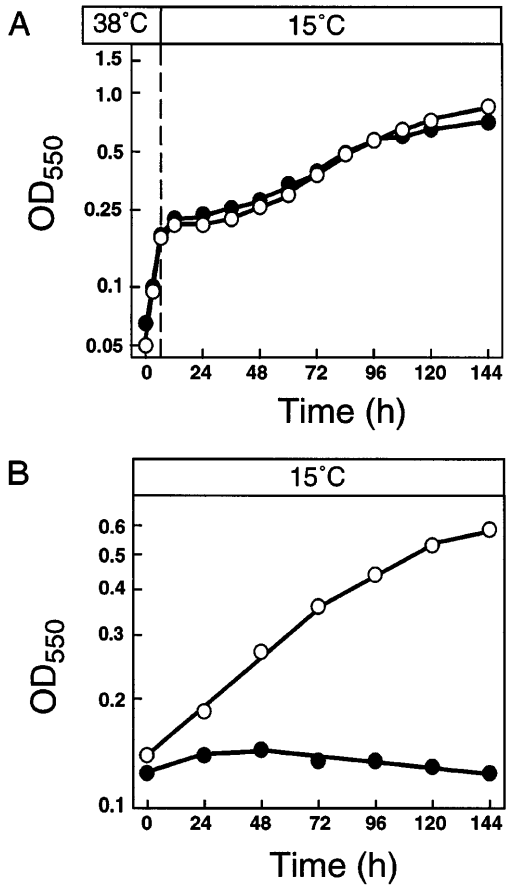


Fig. 3A, B Growth of wild-type strain PR6000 and the *desB* mutant strain PR6081 at 15° C. **A** Wild-type strain PR6000 (○) and the *desB* mutant strain PR6081 (●) were grown at 38° C in medium A^+ containing nitrate as a nitrogen source under 250 μE m^{-2} s^{-1} with aeration by 1% (v/v) CO_2 in air. After growth for 6 h at 38° C, the culture was shifted down to 15° C under otherwise identical growth conditions. Both strains became quite chlorotic (yellow-green) in color during incubation at 15° C (data not shown). **B** Wild-type strain PR6000 (○) and the *desB* mutant strain PR6081 (●) were grown at 15° C in medium A^+ under 250 μE m^{-2} s^{-1} with aeration by 1% (v/v) CO_2 in air. After 138 h of growth at 15° C, cells were inoculated (OD_{550} = approximately 0.1) into fresh medium A^+ and the incubation was continued under identical growth conditions at 15° C

PCC 7002 (PR6000) have their maximum doubling time ~ 4 h) during exponential growth in medium A^+ at 38° C under 250 μE m^{-2} s^{-1}. When wild-type cells growing exponentially at 38° C are transferred to 22° C, the growth rate decreases approximately 2.5-fold to 10 ± 1 h (Fig. 2, PR6000).

The growth rates of the *desA* and *desB* mutant strains PR6080 and PR6081 were identical to that of the wild-type strain at 38° C (Fig. 2, open circles). After a temperature shift-down to 22° C, the growth rate of the *desA* mutant strain PR6080 initially had a value similar to that of wild-type cells, but it reproducibly decreased over a period of approximately 12 h at 22° C (Fig. 2, closed circles). This growth was neither arithmetic nor exponential, but something intermediate. However, after a temperature shift-down to 22° C, the *desB* mutant strain PR6081 had a doubling time similar to that for the wild-type (Fig. 2, closed circles).

When cells growing at 250 μE m^{-2} s^{-1} at 38° C were transferred to 15° C (Fig. 3A), no significant difference was observed in the growth profiles for wild-type strain PR6000 and *desB* mutant strain PR6081. A lag of approximately 30 h was observed before cells grew arithmetically (see Sakamoto and Bryant, 1997b). After cells had acclimated for 138 h at 15° C after the shift-down from 38° C, the ability to grow continuously at 15° C was tested by subculturing cells into fresh medium A^+ (Fig. 3B). Wild-type strain PR6000 continued to grow arithmetically at 15° C, but the *desB* mutant strain PR6081 could not grow after subculturing at 15°C. To test the effects of acclimation to 22° C on a temperature shift-down to 15° C, cells were inoculated into fresh medium A^+ and incubated for 12 h at 22° C prior to a temperature shift-down to 15° C.

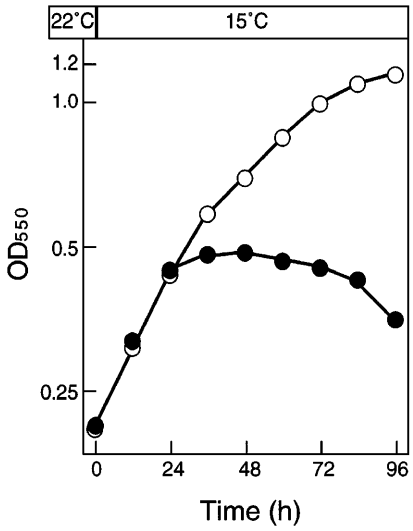


Fig. 4 Effect of temperature shift-down from 22 to 15° C on growth of the *desB* mutant strain PR6081. Wild-type strain PR6000 (○) and strain PR6081 cells (●) were grown at 22° C in medium A^+ containing nitrate as a nitrogen source at 250 μE m^{-2} s^{-1} with aeration by 1% (v/v) CO_2 in air. After 12 h of growth at 22° C, the culture was shifted down to 15° C under otherwise identical growth conditions

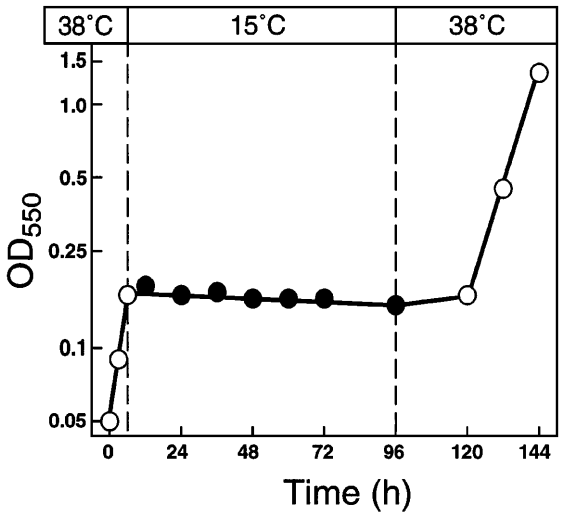


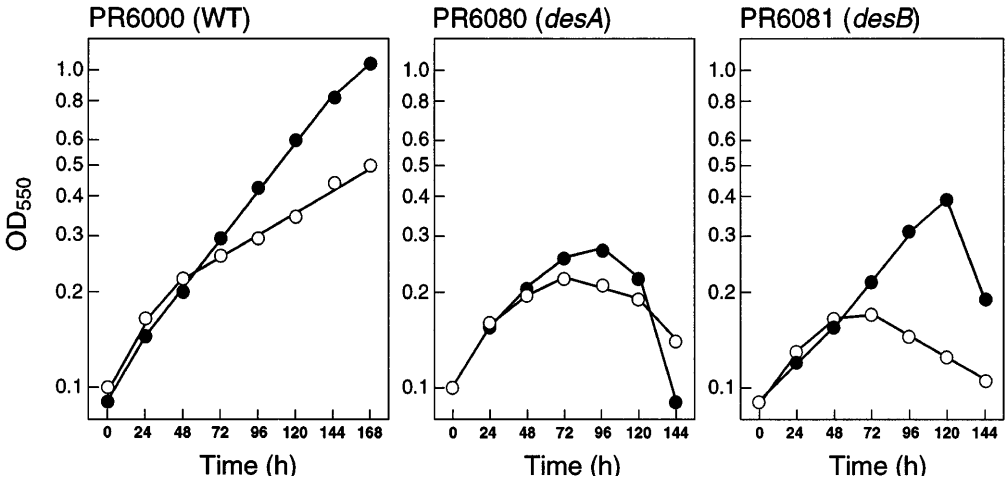
Fig. 5 Effect of temperature shift-down to 15° C on growth of the *desA* mutant strain PR6080. Cells were grown at 38° C in medium A^+ containing nitrate as a nitrogen source at a light intensity of 250 μE m^{-2} s^{-1} with aeration by 1% (v/v) CO_2 in air (○). After 6 h of growth at 38° C, the culture was shifted down to 15° C under otherwise identical growth conditions (●). After a 90-h incubation at 15° C, the culture was shifted back to 38° C

As shown in Fig. 4, cells of wild-type strain PR6000 continued to grow exponentially without a lag phase for a period of about 36 h, after which arithmetic (linear) growth occurred. However, when cells of the *desB* mutant strain PR6081 were similarly acclimated to 22° C and shifted to 15° C, cells grew slowly for a period of about 24 h before all growth stopped (Fig. 4, closed circles).

When cells of *desA* mutant strain PR6080 were subjected to a temperature shift-down from 38° C to 15° C, absolutely no growth occurred at 15° C under 250 μE m^{-2} s^{-1} (Fig. 5). However, if cells that had been held at 15° C for 90 h were returned to 38° C, growth recommenced after a lag of about 24 h and with a doubling time (about 4 h) identical to that of the original growth rate at 38° C. Thus, the *desA* mutant cells retain viability for an extended period of time at 15° C under 250 μE m^{-2} s^{-1} although they are not capable of growth at this temperature. Cells of the *desA* mutant strain PR6080 that had been preacclimated to 22° C for 12 h in medium A^+ under 250 μE m^{-2} s^{-1} likewise did not grow when subjected to a temperature shift-down to 15° C (data not shown). These results show that the *desA* gene product is essential for cell growth at 15° C in medium A^+.

When wild-type cells were grown at 15° C in medium A^+, which contains nitrate as the nitrogen source, the cell coloration became visibly yellow-green because of nitrogen limitation, and growth was arithmetic rather than exponential (Sakamoto and Bryant 1997b). In order to examine the effects of the nitrogen source on the growth of mutant strains PR6080 and PR6081, cells growing at 22° C under 50 μE m^{-2} s^{-1} were transferred to 15° C at the same light intensity in medium A^+ (nitrogen source = nitrate) and in medium A-U (nitrogen source = urea). Wild-type cells (PR6000) in medium A^+ doubled in number by approximately 50 h after the temperature shift-down to 15° C at 50 μE m^{-2} s^{-1}, and the growth rate then slowed and became arithmetic (Fig. 6, PR6000, open circles). During the initial 50-h period after a temperature shift-down to 15° C under 50 μE m^{-2} s^{-1} in medium A^+, the *desA* and *desB* mutants (strains PR6080 and PR6081, respectively) grew similarly to the wild type strain and doubled in number. However, after this initial growth period of approximately 50 h, the *desA* and *desB* mutants entered a death phase in which the optical density decreased with time (Fig. 6, *desA* and *desB*, open circles). The cell coloration of the two mutants changed from the normal blue-green to a chlorotic yellow-green (data not shown), as de-

Fig. 6 Growth of wild-type strain PR6000, *desA* mutant strain PR6080, and *desB* mutant strain PR6081 at 15° C in medium A-U containing urea. Starter cultures were grown in medium A^+ at 22° C under 50 μE m^{-2} s^{-1} with aeration by 1% (v/v) CO_2 in air. The precultures were inoculated into fresh medium A^+ (○) or into fresh medium A-U containing 50 mM urea as a nitrogen source (●). The cultures were incubated at 15° C under otherwise identical growth conditions. All three strains became chlorotic (yellow-green in color) when grown at 15° C in medium A^+, but all strains showed normal blue-green coloration in medium A-U under otherwise identical growth conditions



scribed for wild-type cells grown at 15°C in medium A^+ (Sakamoto and Bryant 1997b). When the *desA* and *desB* mutant cells were shifted back to 38° C under 250 μE m^{-2} s^{-1} after cell growth ceased (after ~ 72 h) at 15° C under 50 μE m^{-2} s^{-1} in medium A^+, normal cell coloration and cell growth quickly recovered after a brief lag phase for both desaturase mutants (data not shown).

When cells grown at 22° C were inoculated into medium A-U containing 50 mM urea at 15° C and 50 μE m^{-2} s^{-1}, wild-type cells grew exponentially with a doubling time of 46 h and achieved a higher cell density than cells growing in medium A^+ (Fig. 6, PR6000, closed circles). Wild-type cells grown in medium A-U at 15° C under 50 μE m^{-2} s^{-1} had normal blue-green coloration and were not nitrogen-starved [see Sakamoto and Bryant (1997b)]. Although the *desA* mutant cells had a normal blue-green color when grown in medium A-U at 15° C and 50 μE m^{-2} s^{-1} (data not shown), these cells could not grow continuously under these conditions (Fig. 6, PR6080, closed circles). Growth gradually ceased and cells then rapidly died. The *desB* mutant strain PR6081 also had normal coloration (data not shown), and growth of this strain was much improved in medium A-U at 15° C and 50 μE m^{-2} s^{-1} (Fig. 6, PR6081, closed circles). However, after a period of ~ 120 h under these conditions, the *desB* mutant cells consistently entered a rapid death phase. Urea is toxic to wild-type cells of *Synechococcus* sp. PCC 7002 and other urease-containing cyanobacteria in stationary phase (T. Sakamoto and D. A. Bryant, unpublished data), and the rapid death phase of the two mutant strains after 120–144 h of incubation in medium A-U could be due to this toxicity effect.

Photosynthesis activity of the desaturase mutants

Table 2 shows the temperature dependency of photosynthetic oxygen evolution rates under saturating actinic light for cells of the wild-type and of the *desA* and *desB* mutant strains grown at 38 and 22° C. No differences were observed in the photosynthetic rates of these three strains when the cells were grown and assayed at 38° C. The oxygen-evolving activity decreased when the assay temperature was lowered to 22 or 15° C, but the assay-temperature dependency of the oxygen evolving activity was similar for each strain. When cells grown at 38° C were assayed at 15° C, the oxygen evolution rate for each strain was approximately 30% of that of the same cells assayed at 38° C. The oxygen evolution rates for all three strains were greatest when cells were grown at 22° C and assayed at 38° C; moreover, the photosynthetic rates for such cells were about twice those of cells grown at 38° C when assayed at 22° C or 15° C. Thus, membrane lipid desaturation at the ω3 position does not seem to affect the acclimative response of the photosynthetic apparatus to growth at 22° C.

Highly variable oxygen evolution rates were obtained with cells of the *desA* mutant strain PR6080 grown at 22°C. The two entries shown in Table 2 reflect the extremes of the data obtained from different cultures of this mutant when grown at 22° C. The temperature dependency of the photosynthetic rate of the *desA* mutant cells was similar to that of wild-type strain PR6000 (i.e., the rate at 15° C was approximately 50% of that for the same cells when assayed at 22° C). This result indicates that desaturation of C_{18} fatty acids at the Δ12 position has little effect on the temperature dependency of the photosynthetic rate. The cause of the variability in the observed photosynthetic rates of the *desA* mutant cells is not

Table 2 Photosynthetic oxygen evolution rates of strains PR6000, PR6080, and PR6081 of *Synechococcus* sp. PCC 7002. The values present the means of at least four experiments. Cells were grown in medium A^+ under 250 μE m^{-2} s^{-1} on 1% CO_2 in air at 38 or 22° C

	Measurement temperature		
	38° C	22° C	15° C
	O_2 evolution, μmol (mg Chl)$^{-1}$ h^{-1} (H_2O to CO_2)		
Grown at 38° C:			
PR6000 (WT)	390 ± 40	230 ± 20	110 ± 10
PR6080 (*desA*)	340 ± 40	210 ± 20	100 ± 20
PR6081 (*desB*)	370 ± 30	200 ± 10	110 ± 20
Grown at 22° C:			
PR6000 (WT)	590 ± 30	400 ± 40	220 ± 20
PR6080 (*desA*)	530 ± 40[a] (290 ± 40)[b]	350 ± 30[a] (240 ± 10)[b]	180 ± 10[a] (120 ± 20)[b]
PR6081 (*desB*)	530 ± 50	450 ± 20	220 ± 10

[a] Activity of *desA* cells was variable; higher-activity cells grown at 22° C (N = 4)
[b] Activity of *desA* cells was variable; lower-activity cells grown at 22° C (N = 7)

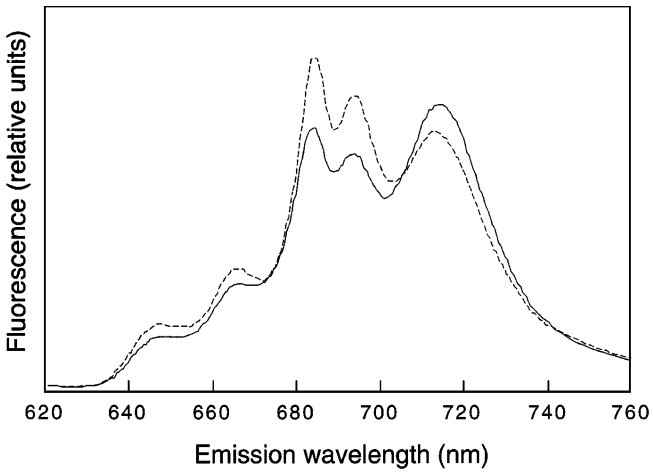


Fig. 7 Fluorescence emission spectra at 77K of whole cells of the *desB* mutant strain PR6081 of *Synechococcus* sp. PCC 7002. The *solid line* (——) is the emission spectrum for cells grown in medium A^+ at 38° C under 250 μE m^{-2} s^{-1} with aeration by 1% (v/v) CO_2 in air. The *dotted line* (----) is the emission spectrum for cells grown at 22° C under otherwise identical growth conditions. The fluorescence emission spectra for wild-type strain PR6000 and the *desA* mutant strain PR6080 were similar to those of strain PR6081 and are not shown. The excitation wavelength was 440 nm

known; however, the length of incubation at 22° C and the apparent severity of nutrient limitation were probably the major contributing factors.

Figure 7 shows the fluorescence emission spectra for whole cells of the *desB* mutant strain PR6081 grown at 38° C (Fig. 7, solid line) and 22° C (Fig. 7, dotted line). When cells were grown at 38° C, the Photosystem-I-associated fluorescence emission peak at 715 nm was greater in amplitude than the Photosystem-II-associated emission peaks at 685 and 695 nm (Fig. 7, solid line). However, in cells grown at 22° C, the amplitudes of the fluorescence emission peaks at 685 and 695 nm were increased relative to the amplitude of the 715-nm emission peak (Fig. 7, dotted line). The fluorescence emission spectra of the *desA* mutant PR6080 (data not shown) were similar to those for the *desB* mutant. These results confirm that membrane lipid desaturation is not involved in the acclimative change in the cyanobacterial photosynthetic apparatus that alters the stoichiometry of Photosystem I and Photosystem II at low temperature (Sakamoto and Bryant 1997b).

Discussion

In this study, mutant strains of *Synechococcus* sp. PCC 7002 specifically defective in fatty acid desaturation at the Δ12 or ω3 (or Δ15) positions were constructed by insertional inactivation of the *desA* (Δ12 acyl-lipid desaturase) and *desB* (ω3 or Δ15 acyl-lipid desaturase) genes. The *desA* mutant strain PR6080 had no observable phenotype at 38° C, but this strain was highly sensitive to low temperature (22° C or less). The *desB* mutant strain PR6081, which is specifically defective in ω3 (Δ15) acyl-lipid desaturation, had no observable phenotype when grown at 22° C or above, but it exhibited low-temperature sensitivity when grown at 15° C. In *Synechococcus* sp. PCC 7002, expression of the *desA* gene occurs at 38° C and is up-regulated at 22° C. Although *desB* mRNA is not detectable at 38° C, this gene is highly expressed at 22° C (Sakamoto and Bryant 1997a; Sakamoto et al. 1997). These results indicate that full expression of the *desA* and *desB* genes occurs at higher temperatures than those at which the absence of their products causes a decline in physiological activities. The rate of enzymatic reactions and energy production must decrease at low temperature, while cells may require additional energy to establish physiological acclimation to low temperature. Our results indicate an effective strategy for solving this paradox: acclimation via acyl-lipid desaturase gene expression occurs well before it becomes necessary.

The rates of biochemical reactions catalyzed by enzymes decrease with decreasing temperature. The Q_{10} value, which is the ratio of the rate constants for a reaction at two temperatures 10° C apart, generally falls between 1.7 and 2.5 for biochemical reactions. The photosynthetic oxygen-evolving activities of the wild-type, *desA*, and *desB* mutant cells decreased identically with decreasing assay temperature, and the temperature dependency of these photosynthetic oxygen-evolving activities was in reasonable agreement with the Q_{10} coefficient for the temperature range of 15–38° C (Table 2). These results imply that lack of membrane lipid desaturation at Δ12 and ω3 positions has no deleterious effect on the photosynthetic apparatus at low temperature, at least within the temporal range of oxygen evolution assays. Thus, the low-temperature sensitivity of cell growth of the *desA* and *desB* mutants is unlikely to result from the inactivation, or even a decrease, of photosynthetic capacity at low temperature.

The *desB* mutant had a growth profile similar to that of the wild type after a temperature shift-down from 38 to 15° C (Fig. 3A). However, after an initial growth period at 15° C, cell growth stopped completely (Figs. 3B and 4). When considered in combination with the photosynthetic oxygen evolution data, these growth studies suggest that *desB* mutant cells stop growing at 15° C because of nutrient limitation. Previous studies have shown that the wild-type strain of *Synechococcus* sp. PCC 7002 becomes nitrogen-limited, chloroses (turns yellow-green in color), accumulates very high levels of glycogen, and exhibits arithmetic rather than exponential growth during incubation in medium A^+ at 15° C (Sakamoto and Bryant 1997b). Since the *desB* mutant cells also became chlorotic during the initial growth period at 15° C, it is likely that much of this initial "growth" of the *desB* mutant at 15° C results from scavenging nitrogenous storage materials from within cells and enhanced light scattering due to glycogen accumulation. Growth of the *desB* mutant at 15° C was improved with urea as a nitrogen source (Fig. 6), and thus it is possible that membrane desaturation at the ω3 position permits the permease used for nitrate assimilation and possibly those for other nutrients to continue to function partially at low temperature. The *desB* mutant cells consistently entered a rapid death phase at 15° C when grown with urea as a nitrogen-source (Fig. 6), suggesting that other nutrient limitation(s) also occurred under these growth conditions for the *desB* mutant. These data show that the disruption of the *desB* gene caused an increase of the lower temperature limit for growth of this cyanobacterium. This is the first report to demonstrate a role of membrane lipid desaturation at the ω3 position on cell growth at low temperature in cyanobacteria.

Although *desA* mutant cells had a photosynthetic rate similar to that of wild-type cells when assayed at 15° C (Table 2), these cells also could not grow at 15° C (Figs. 5, 6). This result implies that the decreased photosynthetic activity of the *desA* mutant is not the limiting factor for growth at 15° C. Under steady-state conditions at 22° C, the *desA* mutant had a significantly slower growth rate than did the wild-type, although the *desA* mutant could grow at a rate similar to that of wild-type cells for a short period immediately following a temperature shift-down to 22° C (Fig. 2). Since *desA* mutant cells had a photosynthetic rate similar to that of wild-type cells at 22° C, it is unlikely that the photosynthetic machinery is damaged at 22° C in *desA* mutant cells. These results suggest that the slower growth rate of the *desA* mutant might be caused by nutrient limitation rather than by a limitation imposed by

the photosynthetic apparatus at low temperature. Nutrient limitation at low temperature might damage many physiological activities of the *desA* mutant cells, and this probably explains the variable results obtained with the *desA* mutant cells grown at 22° C (see Table 2).

It has been proposed that polyunsaturation of membrane lipids enhances the recovery of the Photosystem II protein complex from high-light-induced damage at low temperature in plants and cyanobacteria (Gombos et al. 1992, 1994; Murata and Wada 1995; Nishida and Murata 1996; Tasaka et al. 1996). However, photodamage of the photosynthetic machinery is not likely to limit cell growth at low temperature in the wild-type and in the desaturase mutants of *Synechococcus* sp. PCC 7002. The oxygen evolution activity in wild-type, *desA*, and *desB* cells was stable, and no decline of the oxygen evolution rate at 15° C was observed during oxygen evolution measurements, for which a very high-intensity actinic light (3 mE m^{-2} s^{-1}) was used. Moreover, the *desB* mutant could grow as well as the wild-type strain at 50–250 μE m^{-2} s^{-1} and 15–22° C for several days; thus, it seems unlikely that photodamage of the photosynthetic apparatus limits the growth of this mutant. The results described here and elsewhere (Sakamoto and Bryant 1997b) indicate that repair of photodamage to Photosystem II would decrease at lower temperature because cells become nutrient- (especially nitrogen-) limited, which would in turn slow the rate of PsbA/PsbD protein synthesis.

In cyanobacterial cells, lipids are typically found only in the membranes (Murata and Nishida 1987). Hence, the increased desaturation of lipids at low temperature must represent an environmentally triggered acclimation to improve membrane functionality at low temperature. Previous studies have suggested that irreversible damage to cyanobacterial cells in the dark is initiated at low temperature by a phase separation of plasma membrane lipids (Ono and Murata 1982; Murata and Nishida 1987; Murata 1989). The phase separation of thylakoid membrane lipids occurs at a higher temperature (approximately 25° C in cells of *Anacystis nidulans* grown at 38° C, and 15° C in 28° C-grown cells) than does the phase separation of plasma membrane lipids (15° C in 38° C-grown cells, and 5° C in 28° C-grown cells) (Murata 1989). In this cyanobacterium, phase separation of thylakoid membrane lipids causes reversible loss of photosynthetic activity, and this depression of photosynthetic activity recovers when cells are returned to their growth temperature (Murata 1989).

Since no apparent differences in photosynthetic activities were observed between the wild-type strain and the *desA* and *desB* mutants of *Synechococcus* sp. PCC 7002 in the temperature range of 15–38° C (Table 2), increased desaturation of the lipids of the thylakoid membrane might be less important in the overall acclimation of cells to low temperature than desaturation of the lipids of the plasma membrane. The uptake of nutrients is an important role of the plasma membrane, and our results imply that this function is disrupted when cells are grown at low temperature (or even at higher temperatures when membrane lipid desaturation is reduced).

It has been proposed that the decreased rate of the nutrient uptake from the environment could be the rate-limiting step for growth of microorganisms at low temperature (Nedwell and Rutter 1994). Recent studies with the wild-type strain of *Synechococcus* sp. PCC 7002 have shown that nitrate assimilation becomes limiting for growth at low temperature (Sakamoto and Bryant 1997b). All three enzymes for nitrate assimilation are associated with membranes: the nitrate transporter is integrated in the plasma membrane (Omata 1995), and nitrate and nitrite reductases are components of the thylakoid membrane (Flores and Herrero 1994). It is possible that membrane lipid unsaturation protects these enzymes from inactivation at low temperature. It has been suggested that the nitrate-binding protein NrtA in the ABC transporter of nitrate could be a lipoprotein (Maeda and Omata 1997). Covalent modification with lipids is a common feature of many membrane-associated proteins, and the acyl groups function to anchor such proteins to membranes (Hayashi and Wu 1990). Although the acylation of substrate-binding proteins of ABC transporters is not yet directly demonstrated, it is interesting to speculate that the transporter activity, or even the affinity of the binding protein for substrate, could be controlled by varying the fatty acyl moiety or the local membrane-lipid environments around such transporters.

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References

Cossins AR (1994) Homeoviscous adaptation of biological membranes and its functional significance. In: Cossins AR (ed) Temperature adaptation of biological membranes. Portland Press, London, pp 63–76

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

Flores E, Herrero A (1994) Assimilatory nitrogen metabolism and its regulation. In: Bryant DA (ed) The molecular biology of cyanobacteria. Kluwer, Dordrecht, pp 487–517

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 USA 89:9959–9963

Gombos Z, Wada H, Murata N (1994) The recovery of photosynthesis from low-temperature photoinhibition is accelerated by the unsaturation of membrane lipids: a mechanism of chilling tolerance. Proc Natl Acad Sci USA 91:8787–8791

Harwood JL, Jones AL, Perry HJ, Rutter AJ, Smith KL, Williams M (1994) Changes in plant lipids during temperature adaptation. In: Cossins AR (ed) Temperature adaptation of biological membranes. Portland Press, London, pp 107–118

Hayashi S, Wu HC (1990) Lipoproteins in bacteria. J Bioenerg Biomembr 22:451–471

Maeda S-I, Omata T (1997) Substrate-binding lipoprotein of the cyanobacterium *Synechococcus* sp. strain PCC 7942 involved in the transport of nitrate and nitrate. J Biol Chem 272:3036–3041

Murata N (1989) Low-temperature effects on cyanobacterial membranes. J Bioenerg Biomembr 21:61–75

Murata N, Nishida I (1987) Lipids of blue-green algae (cyanobacteria). In: Stumpf PK (ed) The biochemistry of plants, vol 9. Academic Press, San Diego, pp 315–347

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

Murata N, Wada H, Gombos Z (1992) Modes of fatty-acid desaturation in cyanobacteria. Plant Cell Physiol. 33:933–941

Nedwell DB, Rutter M (1994) Influence of temperature on growth rate and competition between two psychrotolerant antarctic bacteria: low temperature diminishes affinity for substrate uptake. Appl Environ Microbiol 60:1984–1992

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

Omata T (1995) Structure, function and regulation of nitrate transport system of the cyanobacterium *Synechococcus* sp. PCC7942. Plant Cell Physiol 36:207–213

Ono T-A, Murata N (1982) Chilling susceptibility of the blue-green alga *Anacystis nidulans*. 3. Lipid phase of cytoplasmic membrane. Plant Physiol 69:125–129

Russell NJ (1984) Mechanisms of thermal adaptation in bacteria: blueprints for survival. Trends Biochem Sci 9:108–112

Sakamoto T, Bryant DA (1997a) Temperature-regulated mRNA accumulation and stabilization for fatty acid desaturase genes in the cyanobacterium *Synechococcus* sp. strain PCC 7002. Mol Microbiol 23:1281–1292

Sakamoto T, Bryant DA (1997b) Growth at low temperature causes nitrogen limitation in the cyanobacterium *Synechococcus* sp. PCC 7002. Arch Microbiol 169 : 10–19

Sakamoto T, Wada H, Nishida I, Ohmori M, Murata N (1994a) Identification of conserved domains in the Δ12 desaturases of cyanobacteria. Plant Mol Biol 24:643–650

Sakamoto T, Los DA, Higashi S, Wada H, Nishida I, Ohmori M, Murata N (1994b) Cloning of ω3 desaturase from cyanobacteria and its use in altering the degree of membrane-lipid unsaturation. Plant Mol Biol 26:249–263

Sakamoto T, Higashi S, Wada H, Murata N, Bryant DA (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, Murata N (1980) Temperature shift-induced responses in lipids in the blue-green alga, *Anabaena variabilis*: the central role of diacylmonogalactosylglycerol in thermo-adaptation. Biochim Biophys Acta 619:353–366

Stevens SE Jr, Porter RD (1980) Transformation in *Agmenellum quadruplicatum*. Proc Natl Acad Sci USA 77:6052–6056

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

Wada H, Murata N (1990) Temperature-induced changes in the fatty acid composition of the cyanobacterium, *Synechocystis* PCC6803. Plant Physiol 92:1062–1069

Wada H, Gombos Z, Murata N (1990) Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation. Nature 347:200–203

Wada H, Gombos Z, Sakamoto T, Murata N (1992) Genetic manipulation of the extent of desaturation of fatty acids in membrane lipids in the cyanobacterium *Synechocystis* PCC6803. Plant Cell Physiol 33:535–540

Wada H, Gombos Z, Murata N (1994) Contribution of membrane lipids to the ability of the photosynthetic machinery to tolerate temperature stress. Proc Natl Acad Sci USA 91:4273–4277