Temperature-independent and -dependent expression of desaturase genes in filamentous cyanobacterium *Spirulina platensis* strain C1 (*Arthrospira* sp. PCC 9438)

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Abstract

The alteration of the degree of unsaturated fatty acids in membrane lipids has been shown to be a key mechanism in the tolerance to temperature stress of living organisms. The step that most influences the physiology of membranes has been proposed to be the amount of di-unsaturated fatty acids in membrane lipids. In this study, we found that the desaturation of fatty acid to yield the di-unsaturated fatty acid 18:2(9,12), in *Spirulina platensis* strain C1, was not regulated by temperature. As shown by the fatty acid composition and gene expression patterns, the levels of 18:1(9) and 18:2(9,12) remained almost constant either when the cells were grown at 35°C (normal growth temperature) or 22 and 40°C. The expression of *desC* (v9) and *desA* (v12) genes, which are responsible for the introduction of first and second double bonds into fatty acids, respectively, was not affected by the temperature shift from 35 to 22°C or to 40°C. Only the expression and mRNA stability of the *desD* gene (v6) that is responsible for the introduction of a third double bond into fatty acids were enhanced by a temperature shift from 35 to 22°C, but not the shift from 35 to 40°C. The increase in the level of *desD* mRNA elevated the desaturation of fatty acid from 18:2(9,12) to 18:3(6,9,12) at 22°C, indicating a slow response to temperature of fatty acid desaturation in this cyanobacterium. These findings suggest that the desaturation of fatty acids might not be a key mechanism in the response to the temperature change of *S. platensis* strain C1. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Fatty acid desaturation; Gene regulation; Temperature stress; Temperature adaptation

1. Introduction

*Spirulina platensis*, a filamentous cyanobacterium, is widely used as human health food and animal feed. It contains a high level of γ-linolenic acid, 18:3(6,9,12) that is associated with pharmaceuticals and nutraceuticals [1,2]. For example, it can prevent the development of atherosclerosis and coronary heart disease by lowering the level of plasma cholesterol and triglycerides [3]. Therefore, *S. platensis* has been considered to be a new powerful source for γ-linolenic acid production [2,4,5]. γ-Linolenic acid in *S. platensis* is synthesized by the introduction of three double bonds (desaturation) into fatty acids of membrane lipids. The desaturation takes place at the sn-1 position of the glycerol moiety of monogalactosyl diacylglycerol and digalactosyl diacylglycerol. The first double bond is introduced at the Δ9 position of stearic acid (18:0) to yield oleic acid, 18:1(9). The second double bond is then introduced at the Δ12 position of 18:1(9), yielding linoleic acid, 18:2(9,12). Subsequently, the third double bond is added at the Δ6 position of 18:2(9,12) to yield γ-linolenic acid, 18:3(6,9,12). These steps involve three different desaturation enzymes, Δ9, Δ12 and Δ6 desaturases that are encoded by the *desC*, *desA* and *desD* genes, respectively [6]. These three genes were cloned from *S. platensis* strain C1 and their sequences were submitted to EMBL Data Library with the accession numbers, x8673, AJ002065 and x87094 for the *desA*, *desC* and *desD*, respectively.

It is well documented that the content of polyunsatu-
rated fatty acids in membrane lipids of cyanobacteria can be altered by changing the temperature [7–9]. A decrease in temperature increases the unsaturation of fatty acids, whereas an increase in temperature reduces the composition of polyunsaturated fatty acids in membrane lipids. This regulation of fatty acid desaturation is known in term of homeoviscous adaptation or adjustment of membrane fluidity that is necessary to maintain the optimal function of biological membranes [10]. Furthermore, it was demonstrated that the position of double bonds in fatty acids is more influential to the fluidity of membrane lipids than the number of double bonds in fatty acids [11]. It was found that the temperature of the phase transition dramatically decreased when the first and second double bonds were introduced into fatty acids, whereas the introduction of the third and fourth double bonds did not further lower the temperature of phase transition of membrane lipids [12].

The mechanism that regulates the fatty acid desaturation of membrane lipids in response to temperature has been demonstrated to be the result of the up- or down-regulation of the expression of the desaturase genes [13]. As shown in Synechocystis sp. PCC 6803, a decrease in temperature from 34 to 22°C induced the expression of desA, desB (for Δ15 desaturase) and desD genes, but not desC [14,15]. The enhanced levels of desA, desB and desD mRNAs results from two combination effects, the induction of gene expression and the enhancement of mRNA stability [14]. Kis et al. [16] found that the expression of desaturase genes in this strain could be induced by low temperature only in the presence of light. They suggested that the extent of fatty acid desaturation in response to the change of ambient temperature is primarily regulated via light-dependent signaling pathway [16]. In Synechococcus sp. PCC 7002, the expression of the desaturase genes was also regulated by temperature. However, the modes of expression of the genes in this strain were slightly different from that of Synechocystis sp. PCC 6803. The mRNA levels of all desaturase genes, desA, desC and desB, increased when the temperature was shifted from 38 to 22°C. The expression of the desA gene was controlled at the post-transcription level by the enhancement of its mRNA stability. The enhanced level of desC mRNA was due to the increase in the rate of transcription at 22°C. The expression of the desB gene was controlled by a combination of induction of mRNA synthesis and enhancement of mRNA stability [17,18]. It is noteworthy that the features of desaturases of these two unicellular cyanobacterial strains are similar and conserved [13], but the regulatory mechanism of expression of the same desaturase gene is different and depends on the biology of each organism. Therefore, the modes of fatty acid desaturation and expression of desaturase genes in S. platensis is probably different between these two strains.

In order to obtain a greater understanding of fatty acid desaturation in S. platensis, the responses of desaturases to low and high temperatures were examined by following the change in fatty acid composition and expression of the desaturase genes. We found that the response to temperature change of S. platensis strain C1 was different from that of Synechocystis sp. PCC 6803 and Synechococcus sp. PCC 7002. In this strain, the expression of the desA gene which has been shown to be most important for low temperature tolerance was not controlled by temperature. The gene that was responsible for the temperature change in S. platensis strain C1 was the desD gene.

2. Materials and methods

2.1. Organism and culture conditions

S. platensis strain C1 (or Arthospira sp. PCC 9438 as designated by the Pasteur Institute, Paris, France) was grown at 35°C and designated temperatures in Zarrouk’s medium [19] under illumination by fluorescent light at 100 μE m−2 s−1 with continuous stirring. For the temperature-shift experiment, the cells were grown at 35°C up to the exponential phase (optical density at 560 nm was 0.3), then the cells were shifted to 22 or 40°C and incubated for designated periods under the same light conditions.

To determine the half-life of mRNA at 22 and 40°C, rifampicin (Sigma, St. Louis, MO, USA) at a final concentration of 150 μg ml−1 was added to the culture 20 min after the temperature shift. To determine the half-life of mRNA at 35°C, rifampicin at the same concentration was added to the culture 5 min before collecting the cells at time zero.

2.2. Fatty acid analysis

Lipids were extracted from the cells of S. platensis strain C1 according to the method of Bligh and Dyer [20]. The extracted lipids were subjected to methanolysis in 5% HCl in methanol at 85°C for 2.5 h. The resultant fatty-acid methyl esters were analyzed with a gas chromatograph (Shimadzu; GC-9A) equipped with a capillary column (SP 2330, 60 m × 0.25 mm). The relative fatty acid content was determined by comparing their peak areas with that of the internal standard, heptadecanoic acid (Sigma).

2.3. DNA probes

The desA specific probe (387 bp) was generated by PCR using the oligonucleotides 5’CACAGTTTGGCGTATTG-GTC 3’ and 5’GGCGATGGTGAT GAAGT 3’, as forward and reverse primers, and plasmid pBluescript/desA [6] as the template. The desC probe (500 bp) was provided by Dr. D.A. Los (Institute of Plant Biology, Russian Academy of Science). The desD probe (1.2 kb) was excised from the plasmid pBluescript/desD [6] by digestion with the restriction enzyme, HinclII.
2.4. Isolation of total RNA and Northern blot analysis

Cells were harvested by filtration using a magnetic filter funnel (PSF housing and stem 300 ml, Gelman Science, Ann Arbor, MI, USA). Cell pellets were immediately frozen in liquid nitrogen and broken by grinding with a pestle in a cold mortar. Total RNA was extracted in TRIzol reagent as described by the company (Gibco BRL, Life Technologies, Paisley, UK).

For Northern blot analysis, 5–10 µg of total RNA was electrophoresed on 1.2% agarose gel in HEPES/EDTA buffer (50 mM HEPES-NaOH pH 7.8, 1 mM EDTA, 6% formaldehyde) and then transferred onto a nylon membrane (Hybond N, Amersham International, Buckinghamshire, UK). After fixing RNA on the membrane by a UV-crosslinker (UV stratalinker, Stratagene, La Jolla, CA, USA), the membrane was prehybridized in Church and Gilbert solution [21] containing 0.5 M NaH₂PO₄/Na₂HPO₄ (pH 7.2), 1 mM EDTA (pH 8.0) and 7% (w/v) SDS. The ³²P-labeled DNA probe was added and the membrane was hybridized at 45°C for 12 h. The membrane was washed twice with solution I (2× SSC plus 0.1% (w/v) SDS) and solution II (0.5× SSC plus 0.1% (w/v) SDS) at 65°C for 15 min. The size of desaturase mRNA was estimated using an RNA molecular mass marker (Promega, Madison, WI, USA). The level of desaturase mRNA was normalized by comparing the density of the bands with that of 16s rRNA. The density of the bands was estimated by using the image analysis softwares (Adobe version 4.0 and AAB camera scan image program).

3. Results

3.1. Fatty acid composition of total lipids of S. platensis strain C1 grown at isothermal temperature

The major fatty acids found in S. platensis strain C1 were 16:0, 18:2(9,12) and 18:3(6,9,12), whereas 16:1(9), 18:0 and 18:1(9), were present at low levels (Table 1). At 35°C (normal growth temperature), the proportion of 16:0 was 45% of total fatty acids and that of 18:2(9,12) and 18:3(6,9,12) was 21%. At 22°C, the proportion of 16:0 was slightly lower than that of 35°C-grown cells. In contrast, the proportion of 18:3(6,9,12) increased to 27% in the 22°C-grown cells. The proportion of 18:2(9,12) in 22°C-grown cells was approximately 20%. At 40°C, there was no significant change in the proportion of 16:0. The proportion of 18:2(9,12) in 40°C-grown cells was slightly higher than that of 35°C-grown cells, whereas the proportion of 18:3(6,9,12) was 6% lower. When the changes in the proportion of fatty acids at 22 and 40°C were compared, the differences in 16:0 and 18:2(9,12) were within 6%. The greatest change (more than 10%) was observed in the proportion of 18:3(6,9,12). These results indicated that the growth temperature had little effect on the composition of most of the fatty acids in S. platensis strain C1. Only the proportion of 18:3(6,9,12) was significantly altered by growth temperature.

Table 1

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Growth temperature (°C)</th>
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<tr>
<td></td>
<td>22</td>
</tr>
<tr>
<td>16:0</td>
<td>40</td>
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<tr>
<td>16:1(9)</td>
<td>6</td>
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<tr>
<td>18:0</td>
<td>1</td>
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<tr>
<td>18:1(9)</td>
<td>6</td>
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<tr>
<td>18:2(9,12)</td>
<td>20</td>
</tr>
<tr>
<td>18:3(6,9,12)</td>
<td>27</td>
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</table>

The values represent the averages of two independent experiments. The deviation of the values was within ±2%.
3.2. Effect of temperature shift on the composition of fatty acids in *S. platensis* strain C1

The temperature shift from 35 to 22°C (Fig. 1A) did not significantly affect the proportion of 16:0, 16:1(9), 18:0 and 18:1(9). The proportion of 18:2(9,12) slightly decreased from 21 to 19%, whereas the proportion of 18:3(6,9,12) gradually increased from 20% after 36 h of incubation at 22°C to reach 26% at 60 h. The shift from 35 to 40°C (Fig. 1B) affected the proportions of 18:2(9,12) and 18:3(6,9,12). The proportion of 18:2(9,12) increased from 21 to 25% while the proportion of 18:3(6,9,12) decreased from 20 to 12%. These results also indicated that the proportions of most of the fatty acids were not significantly altered by the shift of temperature either from 35 to 22°C or to 40°C. The biggest effect was observed in the proportion of 18:3(6,9,12).

3.3. Effect of temperature shift on the expression of the desaturase genes

To understand the response of *S. platensis* strain C1 to

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Fig. 2. Changes in levels of desaturase mRNAs of *S. platensis* strain C1 after a temperature shift from 35 to 22°C. The cells of *S. platensis* strain C1 were grown at 35°C up to exponential phase then one portion of the cells was harvested (time zero). The remaining culture was shifted to 22°C and incubated according to the indicated period of time (10–120 min). Then the cells were shifted back to 35°C and further incubated at this temperature for 30 min. For the Northern blot analysis, 5 μg of total RNA was electrophoresed and hybridized with a 32P-labeled *desA* probe. The probe was then stripped and the membrane was hybridized with 32P-labeled *desC* and *desD* probes, respectively. (A) Levels of the desaturase mRNAs as determined by Northern blot analysis. (B) Relative changes in mRNA levels of *desC* (○), *desA* (□) and *desD* (▲). The values were obtained after quantification of radiographs.

Fig. 3. Changes in levels of desaturase mRNAs of *S. platensis* strain C1 after a temperature shift from 35 to 40°C. The cells of *S. platensis* strain C1 were grown at 35°C up to an exponential phase then one portion of the cells was harvested (time zero). The remaining culture was shifted to 40°C and incubated according to the indicated period of time (10–120 min). Northern blot analysis was performed the same as described in the legend to Fig. 2. (A) Levels of three desaturase mRNAs as determined by Northern blot analysis. (B) Relative changes in mRNA levels of *desC* (○), *desA* (□) and *desD* (▲). The values were obtained after quantification of radiographs.
temperature change at the molecular level, the modes of expression of three desaturase genes were determined by using Northern blot analysis. Fig. 2 shows the effect of temperature shift from 35 to 22°C on the expression of the desaturase genes. The mRNAs of desC, desA and desD genes were detected at sizes of 1.5, 1.5 and 1.4 kb, respectively. The level of desC mRNA was abundant at 35°C and it remained constant either when the cells were shifted to 22°C or back to 35°C (Fig. 2B). The level of desA mRNA was also abundant at 35°C. Interestingly, the shift to 22°C or back to 35°C did not affect the level of desA mRNA (Fig. 2B). For the desD gene, its mRNA level was low at 35°C. The shift to 22°C increased the level of desD mRNA three-fold within 30 min of incubation and remained constant before cells were shifted back to 35°C, whereby the level of desD mRNA was reduced to 20% of the maximal level (Fig. 2B). These findings indicated that the expressions of desD gene was induced by the downward shift to 22°C.

To examine whether high temperature has any effect on the expression of desaturase genes in S. platensis strain C1, the cells were shifted from 35 to 40°C. The results revealed that the levels of desC, desA and desD mRNAs remained constant (Fig. 3B). This indicated that the expressions of all desaturase genes in S. platensis strain C1 were not affected by an upward shift in temperature.

3.4. Half-life of the desaturase mRNAs

The effect of temperature change on the stability of the three desaturase mRNAs was examined by determining the half-life of mRNAs at 35, 40 and 22°C. The rate of degradation of desC mRNA at each temperature is shown in Fig. 4A. The half-life of desC mRNA at 35 and 40°C was approximately 20 min. At 22°C, it increased from 20 to 30 min (1.5-fold). At 35 and 40°C, the rates of degradation of desA mRNA were similar (Fig. 4B). The half-life of desA mRNA at these two temperatures was approximately 7 min. At 22°C, the half-life of desA mRNA increased twofold (14 min). For the desD gene, the half-life of its mRNA was 5 min at 35°C. At 40°C, the desD mRNA was less than 5 min. At 22°C, the rate of degradation of desD mRNA was reduced seven-fold (half-life of 35 min). These results indicated that the shift to 22°C increased the stability of all desaturase mRNAs, of which the desD mRNA was most significantly affected. In contrast, the temperature shift to 40°C affected only the stability of desD mRNA.

4. Discussion

The effect of low and high temperatures on fatty acid desaturation was studied in S. platensis strain C1 that belongs to cyanobacteria group 3 [22].
The shift of temperature from 35 to 22°C did not affect the expression of desC and desA genes, which are responsible for the introduction of first and second double bonds into fatty acids in membrane lipids (Fig. 2). Even though a slight enhancement of their mRNA stabilities was observed at 22°C (Fig. 4), this effect did not influence the levels of desC and desA mRNAs either when the cells were shifted to 22°C or back to 35°C (Fig. 2). It has been demonstrated that most of the biochemical reactions catalyzed by enzymes decrease with the decreasing temperature [23]. Thus, the increased stability of desC and desA mRNAs in S. platensis strain C1 at 22°C was probably not caused by post transcription control of gene expression, but rather due to a decrease in ribonuclease activity. Coolbear et al. [12] demonstrated that the introduction of the first and second double bonds into di-18:0-synthetic phosphatidylcholines lowered the phase transition temperature at 50 and 22°C, respectively. The introduction of third and fourth double bonds into fatty acids did not reduce the temperature of phase transition [12]. This was correlated with in vivo experiments of Tasaka et al. [24]. The elimination of 18:3(6,9,12) and 18:4(6,9,12,15) by targeted mutagenesis of the desB and desD genes in Synechocystis sp. PCC 6803 had no effect on the growth and phase transition of membrane lipids. In this study, the expression of desC and desA genes of S. platensis strain C1 was not regulated by temperature (Fig. 2). The results from fatty acid analysis were correlated with the gene expression patterns. The levels of 18:1(9) and 18:2(9,12) did not significantly change upon the shift to 22°C (Fig. 1). Our findings strongly suggest that mono- and di-unsaturated fatty acids are essential for the cell growth. Therefore, the constitutive expressions of desC and desA are required for the growth of S. platensis strain C1.

In Synechocystis sp. PCC 6803 and Synechococcus sp. PCC 7002, the desA gene was up-regulated by temperature and also involved in low temperature adaptation of the cells [14,15,17,18], which was different from the desA gene of S. platensis strain C1. The expression of this gene was abundant even at normal growth temperature and it was not up-regulated by low temperature (Fig. 2). Moreover, the level of 18:2(9,12) was unusually high. It was above 20% of total fatty acid at every growth temperature (Table 1 and Fig. 1). This suggests that desA and 18:2(9,12) do not play a role in response and adaptation to low temperature stress in S. platensis strain C1. This is the first evidence demonstrating temperature-independent expression of the desA gene in cyanobacteria.

The expression of the desD gene was induced and its mRNA stability was markedly enhanced by the shift from 35 to 22°C (Fig. 2 and 4). These results indicated that the expression of the desD gene was controlled by temperature at both transcription and post transcription levels. Thus, the increased level of 18:3(6,9,12) at 22°C was the result of up-regulation of the desD gene expression, while the decreased level of 18:2(9,12) was due to the extent of desaturation from 18:2(9,12) to 18:3(6,9,12). However, the accumulation of 18:3(6,9,12) was observed 36 h after the temperature was shifted from 35 to 22°C. This result indicates a slow response to the temperature change of fatty acid desaturation in S. platensis strain C1. In Synechocystis sp. PCC 6803, the desD gene has been proposed to be an optional gene for adaptation to low temperature [14,15]. The increase in the proportion of C18:3 fatty acids has been shown to have less effect on the temperature of phase transition of lipid membranes [12,24]. Based on these findings, the extent in fatty acid desaturation at Δ6 position might not influence the temperature phase transition of lipid membranes in S. platensis strain C1. If the increase in the level of 18:3(6,9,12) does not change the fluidity of lipid membranes, homeoviscous adaptation might not be a key mechanism in the response to the temperature of S. platensis strain C1.

It was also noteworthy that the amounts of 16:1(9), 18:1(9), 18:2(9,12) and 18:3(6,9,12) were over 50% of the total fatty acid content (Table 1). This suggests that S. platensis strain C1 contains more unsaturated fatty acids than saturated fatty acids in the membrane lipids. If the degree of unsaturated fatty acids is related to the fluidity of membranes as proposed [11], the structure of the lipid membranes of S. platensis strain C1 could be stable under a wide range of temperatures below optimum (35°C). As such, the cells of S. platensis strain C1 would be able to tolerate low-temperature stress. But, we found that the growth rate of this strain at 22°C was much lower than that of the cells at 35°C (data not shown). Therefore, it suggests that in S. platensis strain C1, unsaturated fatty acids did not play a major role in low temperature adaptation. There might be other factors playing this role in S. platensis strain C1. Thus, further studies are in progress to clarify this hypothesis.

According to this study, the proportion of 18:2(9,12) increased while the proportion of 18:3(6,9,12) decreased upon shifting the temperature from 35 to 40°C (Fig. 1). At the transcriptional level, the expression of all three desaturase genes were not affected by the temperature shift to 40°C. The degradation rate of desD mRNA increased when the cells were incubated at 40°C, but not that of the desC and desA mRNAs (Fig. 4). These results again demonstrate that the desA gene is a temperature-independent gene and the accumulation of 18:2(9,12) at 40°C was not the result of up-regulation of the expression of desA gene. It was found that the desD mRNA was unstable at 40°C. The stability of mRNA is related to the rate of translation to protein [25]. If the rate of translation is high, mRNA will be occupied by ribosomes that can protect mRNA from ribonuclease [25,26]. Thus, the increase in the degradation rate of desD mRNA was probably due to the suppression of translation since the desaturation to yield 18:3(6,9,12) by Δ6 desaturase was not required at high temperature.
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