

Fatty acid desaturases of cyanobacteria
and the modification of membrane lipid unsaturation.

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Abstract

The molecular-biological aspects of fatty acid desaturases were studied in cyanobacteria.

The previous studies on the biosynthesis of unsaturated fatty acids and physiological roles of membrane lipid unsaturation are reviewed in Chapter 1.

The comparative studies of the $\Delta 12$ desaturase genes (*desA*) of cyanobacteria are described in Chapter 2. The *desA* genes were isolated three strains of cyanobacteria, i.e., *Synechocystis* PCC6714, *Synechococcus* PCC7002 and *Anabaena variabilis*. The conserved domains of the $\Delta 12$ desaturases of cyanobacteria were identified.

The molecular-biological studies of the $\omega 3$ desaturase gene (designated *desB*) are described in Chapter 3. The *desB* gene was isolated from *Synechocystis* PCC6803. The membrane lipid unsaturation at the $\omega 3$ position was altered by the genetic manipulation of the *desB* gene in cyanobacteria.

The isolation and characterization of the $\Delta 9$ desaturase genes (designated *desC*) are described in Chapter 4. The $\Delta 9$ desaturases of cyanobacteria were similar to stearyl-coenzyme A desaturases of mammals and yeast, but not to stearyl-(acyl carrier protein) desaturases of higher plants.

Abbreviations

Lipids

DG, diacylglycerol
DGDG, digalactosyl diacylglycerol
GlcDG, monoglucosyl diacylglycerol
MGDG, monogalactosyl diacylglycerol
PA, phosphatidic acid
PC, phosphatidylcholine
PE, phosphatidylethanolamine
PG, phosphatidylglycerol
PI, phosphatidylinositol
SQDG, sulfoquinovosyl diacylglycerol

Fatty acids

Fatty acids are represented by "X:Y(Z)", containing X carbon atoms with Y double bonds at the position Z counted from the carboxyl terminus.

12:0, lauric acid
14:0, myristic acid
16:0, palmitic acid
16:1(9), palmitoleic acid
16:1(3t), *trans*-3-hexadecenoic acid
16:2(7,10), 7,10-hexadecadienoic acid
16:2(9,12), 9,12-hexadecadienoic acid
16:3(7,10,13), 7,10,13-hexadecatrienoic acid
18:0, stearic acid

18:1(9), oleic acid

18:1(11), *cis*-vaccenoic acid

18:2(9,12), linoleic acid

18:3(9,12,15), α -linolenic acid

18:3(6,9,12), γ -linolenic acid

18:4(6,9,12,15), 6,9,12,15-octadecatetraenoic acid

20:4(5,8,11,14), arachidonic acid

Others

ACP, acyl carrier protein

CoA, coenzyme A

IPTG, isopropyl-1-thio- β -D-galactoside.

Km^r, kanamycin-resistance gene

Chapter 1

General introduction.

Biological aspects of cyanobacteria

Cyanobacteria are prokaryotic algae, which can carry out oxygenic photosynthesis, using two photosynthetic reaction center assemblies (photosystem I and II) linked by a cytochrome-containing electron transport chain. The cyanobacterial photosynthetic apparatus is very similar to its counterpart in plant chloroplasts. It is argued that cyanobacteria were the first organisms which were capable of oxidizing water to produce molecular oxygen, and that they were developed about 3×10^9 years ago. Geochemical and fossil evidences suggest that cyanobacteria caused the transition of the global atmosphere of the earth from its primordial, anaerobic state to its current, aerobic condition between 0.5 and 1.5×10^9 years ago (Schopf et al. 1983).

Cyanobacteria are recognized as a major and diverse group in the prokaryote kingdom judged by large variety and large number of species. They include all prokaryotes capable of oxygen-evolving photosynthesis (Whitton and Carr 1982). Based on the type of cell wall, cyanobacteria can be classified as Gram-negative bacteria, of which the cell wall is composed of the outer and plasma (inner) membranes separated by a peptidoglycan layer (Drews and Weckesser 1982). In addition, cyanobacteria have intracellular photosynthetic membranes, that is, the thylakoid membranes. The membrane architecture of the cyanobacterial cells is similar to that of eukaryotic plant chloroplast, which

contains the outer and inner envelope membranes, surrounding the thylakoid membranes.

Cyanobacteria are morphologically divided into two groups, i.e., unicellular and filamentous strains. The dominant nutritional mode of all cyanobacteria is photoautotrophy. However, there are variations in facultative ability to grow on organic substances in the light (photoheterotrophy) or dark (chemoheterotrophy), and to survive in the dark by fermentation or anaerobic respiration (Doolittle 1982). Some strains have the ability to use sulfide or H₂ as electron donor in anoxygenic photosynthesis (Doolittle 1982). In addition, a number of cyanobacteria can convert atmospheric N₂ to ammonium. Heterocysts of filamentous cyanobacteria are differentiated cells which are specialized for aerobic fixation of N₂ (Wolk 1982). When motile, cyanobacteria move by gliding. Gliding occurs at nearly all time for many filamentous members of the Oscillatoriaceae (Castenholz 1982). However, the mechanism of gliding is unsolved so far.

Certain strains of cyanobacteria are naturally transformed with DNA exogenously added to the growth medium (Golden et al. 1987, Haselkorn 1991, Elhai et al. 1990). Three unicellular strains in which the transformation systems have been characterized are *Synechococcus* PCC7942 (*Anacystis nidulans* R2), *Synechococcus* PCC7002 (*Agmenellum quadruplicatum* PR6) and *Synechocystis* PCC6803. In these strains, a cloned cyanobacterial DNA fragment is efficiently directed to a specific region of the cyanobacterial

chromosome by homologous recombination. Several shuttle vectors, which are capable of replication in *Escherichia coli* and a cyanobacterium, can be used to introduce a foreign DNA into cyanobacterial cells. In addition, filamentous cyanobacteria (i.e. several strains of *Anabaena*) has been transformed by conjugal gene transfer. These properties offer a great promise for the identification and manipulation of genes in cyanobacteria.

Recent advances in molecular biology and genetics of cyanobacteria provide novel experimental approaches to study unique physiological and developmental properties of this group of organisms. One of the most spectacular advances is in unraveling the gene expression involved in the control of N₂ fixation and heterocyst development (Golden et al. 1985, Golden and Wiest 1988, Elhai and Work 1990, Liang et al. 1992, Liang et al. 1993). Another impressive advance is the isolation and analysis of the genes of photosynthetic apparatus, the sequences of which provide the information about the structure and evolution of the photosynthetic apparatus. In addition, physical maps, on which all of the currently identified genes can be located, have been obtained for a filamentous cyanobacterium, *Anabaena* PCC7120 (Bancroft et al. 1989) and unicellular one, *Synechococcus* PCC7002 (Chen and Widger 1993). The next goal in cyanobacterial genetics is to complete the total nucleotide sequence of the genome.

Glycerolipids and fatty acids in cyanobacteria

(1) Glycerolipids

Cyanobacteria contain four major glycerolipids, i.e., MGDG, DGDG, SQDG and PG, and a minor component, GlcDG, but not PC, PI, PE, phosphatidylserine nor cardiolipin (Murata and Nishida 1987). The molecular structures of these glycerolipids of cyanobacteria are shown in Fig. 1-1. MGDG represents ca. 50% of total glycerolipids, and DGDG, SQDG and PG are of 10-20%. The GlcDG content apparently does not exceed 1% (Feige et al. 1980, Sato and Murata 1982a). On the other hand, chloroplasts of higher plants contain MGDG, DGDG, SQDG and PG as major components, and PC and PI as minor components (Harwood 1980).

The GlcDG is ubiquitously present in cyanobacteria, but not in eukaryotic algae, and is a precursor in the biosynthesis of MGDG in cyanobacteria (Murata and Nishida 1987). Sato and Murata (1982a) showed by pulse-labeling experiments in *Anabaena variabilis* that the conversion from GlcDG to MGDG results from epimerization of glucose to galactose. They further demonstrated that DGDG is produced by transfer of newly synthesized galactose to MGDG (Sato and Murata 1982a). SQDG and PG are also rapidly labeled, suggesting that these lipids are directly synthesized, but not via GlcDG (Sato and Murata 1982a). Sato and Murata (1982c) also observed, in *Anabaena variabilis*, a membrane-associated activity of UDP-glucose:diacylglycerol glucocyltransferase which synthesizes GlcDG by transfer of

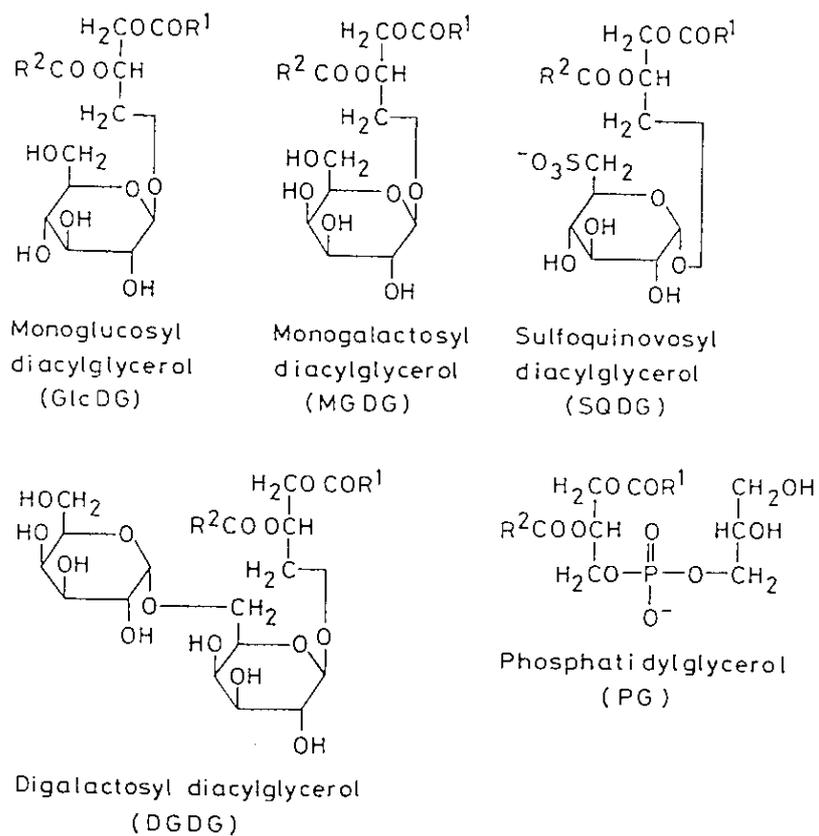


Figure 1-1. Chemical structures of glycerolipids of cyanobacteria (Murata and Nishida 1987). R¹ and R² are acyl groups.

glucose from UDP-glucose to diacylglycerol. It is likely that the GlcDG synthesis activity is located in both thylakoid and plasma membranes of *Anacystis nidulans* (Omata and Murata 1986).

The activities of glycerol-3-phosphate acyltransferase and 1-acylglycerol-3-phosphate acyltransferase, which involved in the first and the second steps of glycerolipid synthesis, have been characterized in crude extract of *Anabaena variabilis* (Lem and Stumpf 1984b).

A tentative scheme for glycerolipid biosynthesis in cyanobacterial cells, based on limited information, is presented in Fig. 1-2.

(2) Fatty acids

The fatty acids so far known to be present in cyanobacteria are 16:0, 16:1(9), 16:2(9,12), 18:0, 18:1(9), 18:1(11), 18:2(9,12), 18:3(9,12,15), 18:3(6,9,12) and 18:4(6,9,12,15). Kenyon (1972) and Kenyon *et al.* (1972) classified the cyanobacteria into four groups by reference to their fatty acid components. Strains in Group 1 contain only saturated and monounsaturated fatty acids, such as 16:1(9) and 18:1(9), whereas strains in the other groups contain polyunsaturated fatty acids. Group 2 is characterized by the presence of 18:3(9,12,15), Group 3 by 18:3(6,9,12), and Group 4 by 18:4(6,9,12,15).

The mode of desaturation of fatty acids in the glycerolipids of cyanobacteria was studied by analyzing the composition of fatty acids, the distribution of fatty acids

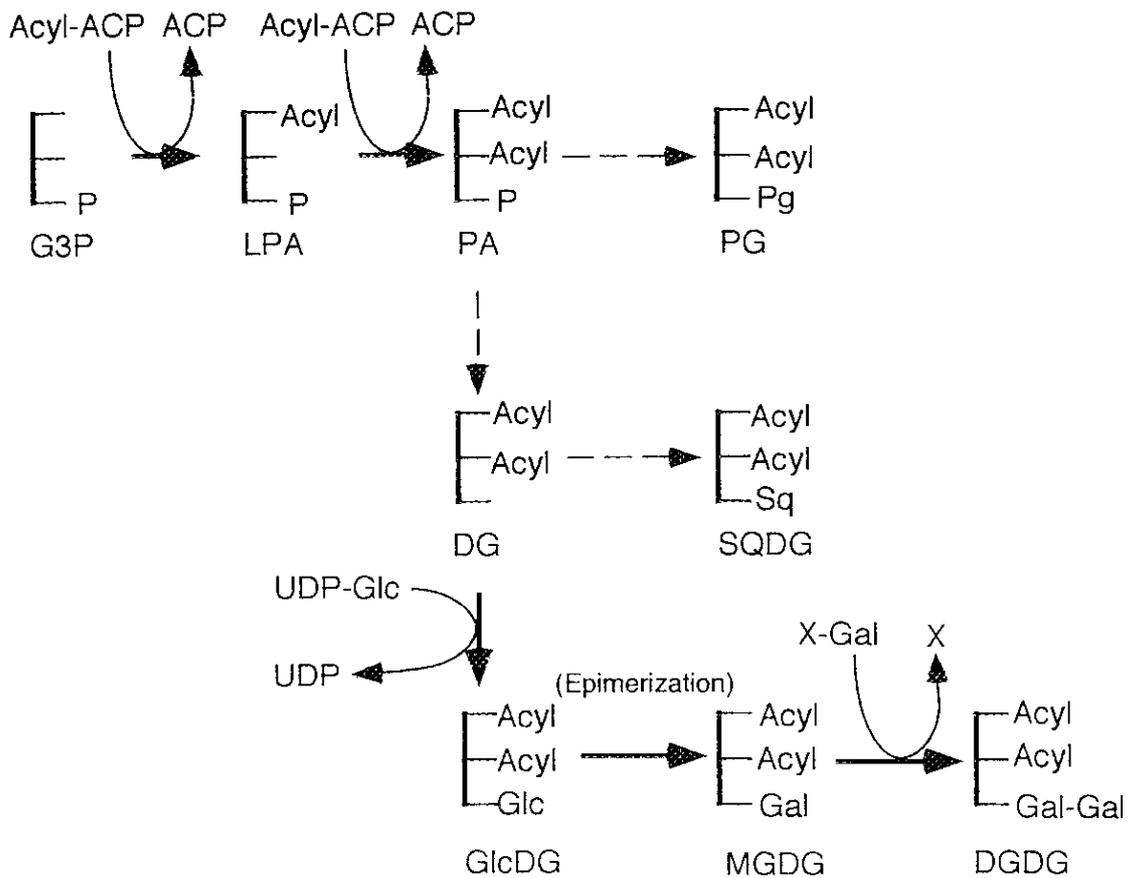


Figure 1-2. A scheme for glycerolipid biosynthesis in cyanobacteria (Murata and Nishida 1987). The reaction indicated by broken arrows have not been experimentally demonstrated. G3P, glycerol 3-phosphate; LPA, 1-acyl-glycerol 3-phosphate (lysophosphatidic acid); Pg, phosphoglycerol; Sq, sulfoquinovose; Glc, glucose; Gal, galactose; X, unidentified galactose carrier.

at the *sn* position of the glycerol moiety, and the position of double bonds in the fatty acids (Murata et al. 1992b). Table 1-1 summarizes the mode of desaturation of fatty acids in cyanobacteria. In Group 1, both 16:0 and 18:0 at the *sn*-1 position are desaturated at the $\Delta 9$ position of the fatty acids. At the *sn*-2 position, 16:0 is desaturated at its $\Delta 9$ position in MGDG and DGDG. Cyanobacteria in Groups 2, 3 and 4 are characterized by a unique positional distribution of fatty acids; the C₁₈ fatty acids are esterified to the *sn*-1 position of the glycerol moiety and the C₁₆ fatty acids to the *sn*-2 position. In Group 2, the C₁₈ acid at the *sn*-1 position is desaturated at the $\Delta 9$, $\Delta 12$ and $\Delta 15$ positions in all lipid classes, and the C₁₆ acid at the *sn*-2 position is desaturated at the $\Delta 9$ and $\Delta 12$ positions of MGDG and DGDG. In Group 3, the C₁₈ acid at the *sn*-1 position is desaturated at the $\Delta 6$, $\Delta 9$ and $\Delta 12$ positions of MGDG and DGDG, and at the $\Delta 9$ and $\Delta 12$ positions of SQDG and PG. In Group 4, the C₁₈ acid at the *sn*-1 position is desaturated at the $\Delta 6$, $\Delta 9$, $\Delta 12$ and $\Delta 15$ positions of MGDG and DGDG, and at the $\Delta 9$, $\Delta 12$ and $\Delta 15$ positions of SQDG and PG. Both unicellular and filamentous strains are distributed among all four groups (Murata et al. 1992b).

Biosynthesis of unsaturated fatty acids in cyanobacteria

The biosynthesis of fatty acids was studied first in *Anabaena variabilis*, which belongs to Group 2 in the mode of fatty acid desaturation, by pulse-labeling with H¹⁴CO₃⁻ and a

Table 1-1. Mode of fatty acid desaturation in cyanobacteria (Murata *et al.* 1992b).

Classification	Δ position of desaturation			
	MGDG and DGDG		SQDG and PG	
	<i>sn</i> -1 (C ₁₈)	<i>sn</i> -2 (C ₁₆)	<i>sn</i> -1 (C ₁₈)	<i>sn</i> -2 (C ₁₆)
Group 1	9 ^a	N ^b or 9	9 ^a	N
Group 2	9,12,15	9,12	9,12,15	N
Group 3	6,9,12	N	9,12	N
Group 4	6,9,12,15	N	9,12,15	N

^a Mixture of C₁₈ and C₁₆ fatty acids.

^b N stands for no desaturation at the *sn*-2 position.

subsequent chase (Sato and Murata 1982b). The primary products of lipid biosynthesis were 1-stearoyl-2-palmitoyl species of GlcDG, SQDG, and PG. They were converted to unsaturated molecular species by desaturation of fatty acids. In GlcDG, the desaturation of fatty acids and epimerization of glucose to galactose is likely to take place independently. DGDG is produced by transfer of galactose from an unidentified galactose carrier to MGDG, and apparently no desaturation of fatty acids take place in DGDG. Nichols (1968) showed that in *Anacystis nidulans*, which contains no polyunsaturated fatty acids (Group 1), also saturated fatty acid are esterified into glycerolipids and then desaturated.

The lack of fatty acid desaturation in the form of acyl-ACP or acyl-CoA was also suggested by *in vitro* experiments. None of 18:0-ACP and 18:0-CoA desaturase activities was detected in any fractions from *Anabaena variabilis* (Lem and Stumpf 1984a, Stapleton and Jaworski 1984). Another cyanobacterium tested, a species of *Nostoc*, did not have 18:0-ACP desaturase activity neither (Lem and Stumpf 1984a). The absence of 18:0-ACP desaturase may be extended to all cyanobacterial strains.

Wada et al. (1993) demonstrated that the fatty acid desaturation activities were associated with membranes isolated from *Synechocystis* PCC6803. The fatty acids at the *sn*-1 position of MGDG were labeled *in vitro* by ¹⁴C-labeled 18:0-ACP and lyso-MGDG using an acyl-ACP:lyso-MGDG acyltransferase activity in the membranes. They

demonstrated the conversion of 18:0 to 18:1(9) at the *sn*-1 position of MGDG. They also observed that the activity of the desaturation of 18:1(9) to 18:2(9,12) was bound to the membranes. These desaturation required the reduced form of ferredoxin.

Substrate specificities of desaturases and acyltransferases were studied by feeding the cyanobacterium, *Synechocystis* PCC6803, with heptanoic acid, 7:0 (Higashi and Murata 1993). This aliphatic acid was elongated to C₁₅, C₁₇ and C₁₉ fatty acids, and incorporated into glycerolipids. These fatty acids were desaturated at the Δ₆, Δ₉, Δ₁₂ and ω₃ positions. This result suggests that the Δ₉ desaturase counts the carbon number from the carboxyl terminus of fatty acids, whereas the ω₃ desaturase, which converts 18:2(9,12) to 18:3(9,12,15), and 18:3(6,9,12) to 18:4(6,9,12,15), does so from the methyl terminus. In the distribution of fatty acids at the *sn*-positions of glycerol moiety, the C₁₇, C₁₈ and C₁₉ fatty acids were located at the *sn*-1 position, whereas the C₁₅ and C₁₆ fatty acids were at the *sn*-2 position. This result suggests that glycerol-3-phosphate acyltransferase specifically transfers 17:0, 18:0 and 19:0, whereas 1-acylglycerol-3-phosphate acyltransferase specifically transfers 15:0 and 16:0.

Some species, such as *Anacystis nidulans*, contain high proportions of 18:1(11). This fatty acid is known to be produced by the anaerobic pathway in bacteria as described later. It is uncertain if these species of cyanobacteria synthesized 18:1(11) as the acyl-ACP form and then esterify

it to the glycerol moiety of glycerolipids (Murata and Nishida 1987).

Role of membrane lipid unsaturation in cyanobacteria

(1) Low-temperature effects of fatty acid unsaturation
Changes in the unsaturation of membrane lipids induced by ambient low temperature have been studied in *Anabaena variabilis* (Sato and Murata 1980, 1981, 1982b) and *Synechocystis* PCC6803 (Wada and Murata 1990). Figure 1-3 shows a hypothetical pathway of lipid synthesis in *Anabaena variabilis* and *Synechocystis* PCC6803.

When the growth temperature is suddenly shifted downward, the fatty acid composition of *Anabaena variabilis* is rapidly altered (Sato and Murata 1980). For 10 h after the temperature shift from 38 to 22°C, the total amount of lipids stays at a constant level (Sato and Murata 1980), but a decrease in 16:0 and a concomitant increase in 16:1(9) take place at the *sn*-2 position of MGDG (indicated by asterisks in Fig. 1-3A). The conversion of 16:0 to 16:1(9) at the *sn*-2 position of MGDG takes place in the lipid-bound form (Sato et al. 1986). Then, as fatty acid and lipid synthesis resumes, the ratio of 16:0 to 16:1(9) is slowly restored to that seen prior to the temperature shift. The transient desaturation is suppressed by chloramphenicol (a protein synthesis inhibitor) and rifampicin (an RNA synthesis inhibitor). The results suggest that a fatty acid desaturase, specific to 16:0 at the *sn*-2 position of MGDG, is transiently synthesized after the downward shift in

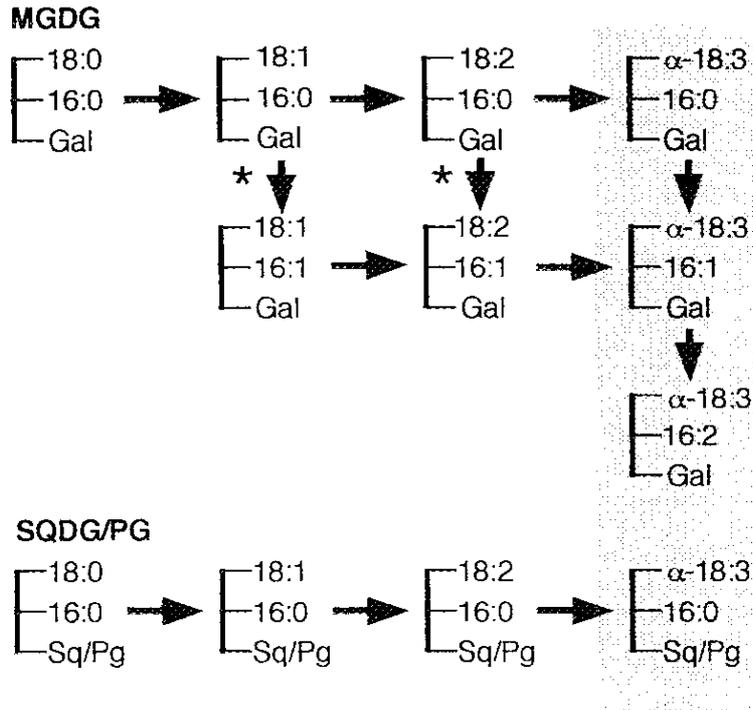
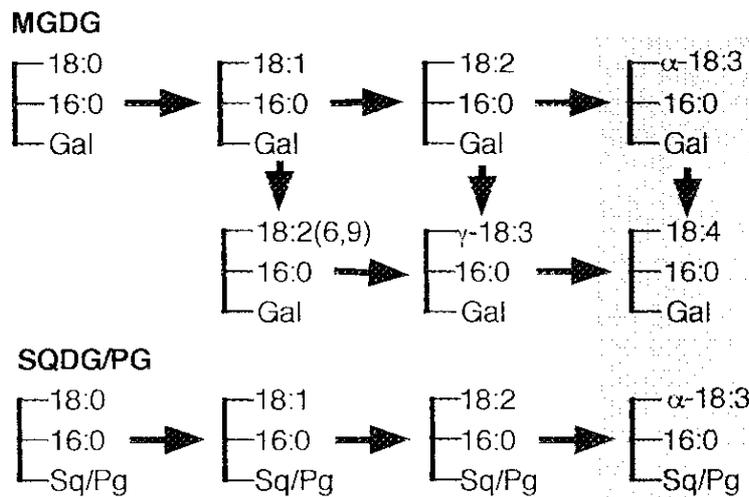
(A) *Anabaena variabilis***(B) *Synechocystis* PCC6803**

Figure 1-3. Schemes for the biosynthesis of glycerolipid molecular species in *Anabaena variabilis* (A) (Sato and Murata 1982b) and *Synechocystis* PCC6803 (B) (Wada and Murata 1990). The glycerolipid molecular species, which are synthesized at low temperature, are indicated by shadowing. The desaturation reaction induced after the downward temperature shift is indicated with asterisk. Pg, phosphoglycerol; Sq, sulfoquinovose; Gal, galactose; α -18:3, α -linolenic acid; γ -18:3, γ -linolenic acid.

temperature (Sato and Murata 1981). Decreases in the contents of 18:1(9) and 18:2(9,12) and an increase in 18:3(9,12,15) take place in MGDG, SQDG and PG after the downward shift in temperature, but these occur more slowly than the changes in C₁₆ acids of MGDG (Sato and Murata 1980).

When the growth temperature of *Synechocystis* PCC6803 is shifted from 34°C to 22°C, total amount and composition of lipids stays constant for 10 h. During this period, however, 18:2(9,12) and 18:3(6,9,12) are desaturated to 18:4(6,9,12,15) at the *sn*-1 position MGDG, and 18:1(9) and 18:2(9,12) are desaturated to 18:3(9,12,15) at the *sn*-1 position of PG and SQDG, while the fatty acids of DGDG are unchanged. In contrast to the C₁₈ acids, the 16:0 at the *sn*-2 position is not desaturated in any of the lipid classes (Wada and Murata 1990). The findings that the desaturation of C₁₈ acid was inhibited by chloramphenicol and rifampicin suggest that desaturase activities are induced after the downward shift in temperature (Wada and Murata 1990).

(2) Mechanism of chilling injury of cyanobacteria

Ono and Murata (1981a,b) have proposed the mechanism for the chilling injury of *Anacystis nidulans*. At chilling temperature, the bilayer lipids of the cytoplasmic membrane are in the phase separated state, which induces leakage of ions and solutes, having low molecular weights, to the outer medium. The decreases in the intracellular concentrations of ions and solutes results in irreversible inactivation of

the physiological activities in the cells. Vigh et al. (1985) confirmed the suggestion that the irreversible damage at low temperature in *Anacystis nidulans* is induced by a phase transition in the plasma membrane, but not in the thylakoid membrane. Selective hydrogenation of the unsaturated fatty acids in the plasma membrane using a chemical catalyst resulted in parallel shift in the degree of fatty acid saturation, the temperature for the onset of phase separation in the plasma membrane, and the temperature critical for ion leakage and the irreversible decline of photosynthesis (Vigh et al. 1985).

The phase separation temperature of membrane lipids will drop with increase in unsaturation of fatty acids esterified to lipids. Thus, the changes in fatty acid composition with growth temperature, as described above, can be regarded as an adaptive response to changes in the ambient temperature (Ono and Murata 1982, Murata et al. 1984). The control of the level of unsaturated fatty acids in the membrane lipids at low temperature should be contributed to the regulation of the fluidity of biological membranes.

Molecular biological studies of fatty acid desaturase in cyanobacteria

In the cyanobacterium, *Synechocystis* PCC6803 has four distinct desaturases, each of which catalyzes the desaturation at the $\Delta 9$, $\Delta 12$, $\Delta 6$ or $\omega 3$ position of fatty acids esterified at the *sn*-1 position of the glycerol

moiety. The gene (*desA*) for $\Delta 12$ desaturase of *Synechocystis* PCC6803 was isolated by complementation of a mutant (*Fad12*) of the cyanobacterium, which was defective in desaturation of fatty acids at the $\Delta 12$ position (Wada and Murata 1989, Wada et al. 1990). The $\Delta 6$ desaturase gene was isolated by expression of a *Synechocystis* genomic cosmid library in *Anabaena* PCC7120, which lacks the desaturation at the $\Delta 6$ position (Reddy et al. 1993). The author isolated the $\omega 3$ desaturase gene (*desB*) and the $\Delta 9$ desaturase gene (*desC*) from *Synechocystis* PCC6803 in the present study as described in Chapter 3 and Chapter 4, respectively.

As described above, the activities of desaturation of fatty acids are induced after the downward shift in ambient temperature. Los et al. (1993) characterized the temperature-dependent regulation of the transcription of the *desA* gene. The level of *desA* transcript increased 10-fold within 1 h upon a decrease in temperature from 36°C to 22°C. The accumulation of the *desA* transcript depend on the extent of temperature change over a certain threshold level, but not on the absolute temperature. The selective hydrogenation of plasma membrane lipids also stimulated the transcription of the *desA* gene (Vigh et al. 1993). These results suggest that the cyanobacterial cells perceive a decrease in the fluidity of plasma membrane lipids when it is exposed to a decrease in temperature.

The role of membrane lipid unsaturation in chilling-acclimation has been extensively studied using genetically manipulated strains of cyanobacteria (Wada et al. 1990,

Gombos *et al.* 1992, Wada *et al.* 1992). Disruption of the *desA* gene in *Synechocystis* PCC6803 results in the lack of the desaturation of fatty acids at the $\Delta 12$ position and makes the cyanobacterium be sensitive to low temperature (Wada *et al.* 1992). At 34°C, the mutant, in which the *desA* gene was disrupted, grew at the same rate as wild-type cells, but the growth rate at 22°C of the mutant was lower than that of wild type. In addition, the mutant decreased tolerance to photoinhibition of photosynthesis at low temperature (Wada *et al.* 1992). Introduction of the *desA* gene into *Synechococcus* PCC7942, a cyanobacterium containing no polyunsaturated fatty acids, rendered this strain to desaturate fatty acids at the $\Delta 12$ position and increased the tolerance of photosynthetic activity to low temperature (Wada *et al.* 1990). Another transformant of *Synechocystis* PCC6803, which was defective in both $\Delta 6$ and $\Delta 12$ desaturation, was more susceptible to low-temperature photoinhibition of photosynthesis than the mutant lacking the $\Delta 12$ desaturation only (Gombos *et al.* 1992). These results suggest that the polyunsaturated fatty acids are necessary for growth and tolerance to photoinhibition of photosynthesis at low temperature.

Biosynthesis of unsaturated fatty acid in higher plants

The *de novo* synthesis of C₁₆ and C₁₈ fatty acids takes place in the plastids of both leaves and developing seeds (Stumpf 1980, 1984, 1987). Acetyl-CoA and malonyl-CoA precursors

are used to synthesize fatty acids to 16:0 as their ACP derivatives. A part of 16:0-ACP is elongated to 18:0-ACP, and rapidly converted to 18:1-ACP by a soluble 18:0-ACP desaturase (Stumpf 1987, Jaworski 1987). The acyl-ACP products of fatty acid synthesis, such as 16:0-ACP and 18:1-ACP, are utilized in plant cells for the biosynthesis of glycerolipids and the associated production of polyunsaturated fatty acids. Although 18:0 is desaturated to 18:1(9) in the ACP-bound form within plastids, all the other desaturations take place in a lipid-bound form (Jaworski 1987, Harwood 1988).

There are two distinct pathways in biosynthesis of polyunsaturated fatty acids bound to glycerolipids in plant cells: the cytoplasmic (eukaryotic) pathway and the plastidial (prokaryotic) pathway (Roughan and Slack 1982).

In the cytoplasmic pathway, acyl groups are exported from plastids as CoA esters, and are used for the synthesis of PA in the endoplasmic reticula. The glycerol-3-phosphate acyltransferases of the endoplasmic reticula synthesize PA which is highly enriched with C₁₈ fatty acids at the *sn*-2 position and is esterified by 16:0 and 18:1(9) at the *sn*-1 position (Roughan 1987). This PA gives rise to the phospholipids, such as PC, PE and PI, which are characteristic of extrachloroplast membranes. 18:1(9) esterified to the *sn*-1 and *sn*-2 positions of PC is desaturated to 18:2(9,12) and further to 18:3(9,12,15) (Jaworski 1987). On the other hand, 16:0 and 18:0 are not desaturated. In addition, the diacylglycerol moiety of PC

is transported back to the chloroplast envelope and contributes the synthesis of a part of the plastid lipids.

By contrast, the PA synthesized in the plastidial pathway has 16:0 at the *sn*-2 position and 18:1(9) at the *sn*-1 position, because of the substrate specificities of the plastid acyltransferases (Frentzen et al. 1983). The PA is used for the synthesis the plastid lipids, i.e., MGDG, DGDG, SQDG and PG. 16:0 is desaturated only in the plastidial pathway. The 16:0 bound to the *sn*-2 position of PG is desaturated to 16:1(3t), and that bound to the *sn*-2 position of MGDG is desaturated to 16:3(7,10,13).

In many species of higher plants, PG is the only product of the plastidial pathway and most of the remaining chloroplast lipids are synthesized by the cytoplasmic pathway. But in some species, such as *Arabidopsis*, both pathways contribute about equally to the synthesis of MGDG, DGDG and SQDG (Browse et al. 1986b). In these plants, the chloroplast lipids contain substantial amounts of 16:3(7,10,13) which is found only in MGDG and DGDG that are produced in the plastidial pathway. These plants are termed "16:3 plants" distinguished them from the other angiosperms, i.e., "18:3 plants".

Mutants in lipid biosynthesis of *Arabidopsis*

An extensive collection of mutants with alteration in lipid composition has been isolated in *Arabidopsis thaliana* (Somerville and Browse 1991, Ohlrogge et al. 1991). These mutants with modified fatty acid composition are due to

single, recessive nuclear mutations. The sites of the enzymatic lesions in seven classes of these mutants (designated *act1*, *fadA*, *fadB*, *fadC*, *fadD*, *fad2* and *fad3*) are tentatively determined as illustrated in Fig. 1-4.

The *act1* mutants are deficient in polyunsaturated C₁₆ fatty acids at the *sn*-2 position of MGDG, which are synthesized only in the plastidial pathway (Kunst *et al.* 1988). This phenotype is due to the deficiency in plastid glycerol-3-phosphate acyltransferase, the first enzyme of the plastidial pathway. As a result of this mutation, all of chloroplast lipids, except for PG, are derived from the cytoplasmic pathway. Nishida *et al.* (1993) isolated the gene (designated *ATS1*) for the plastid-located glycerol-3-phosphate acyltransferase from *A. thaliana*.

The other six mutants are defective in the desaturation of lipid-bound fatty acids. The *fadA* mutants are specifically defective in the desaturation of 16:0 to 16:1(3t) at the *sn*-2 position of PG in the chloroplasts (McCourt *et al.* 1985). Similarly, the *fadB* mutation depletes the desaturation of 16:0 to 16:1(7) at the *sn*-2 position of MGDG in the chloroplasts (Kunst *et al.* 1989). By contrast, the other desaturases (under control by the *fadC*, *fadD*, *fad2* and *fad3*) are not specific for the length of fatty acids, the position of fatty acids on the glycerol moiety, or the nature of the head groups of lipids. The *fadC* mutants are defective in the desaturation of 18:1(9) and 16:1(7) to 18:2(9,12) and 16:2(7,10) in the chloroplasts (Browse *et al.* 1989), the *fadD* in that of 18:2(9,12) and

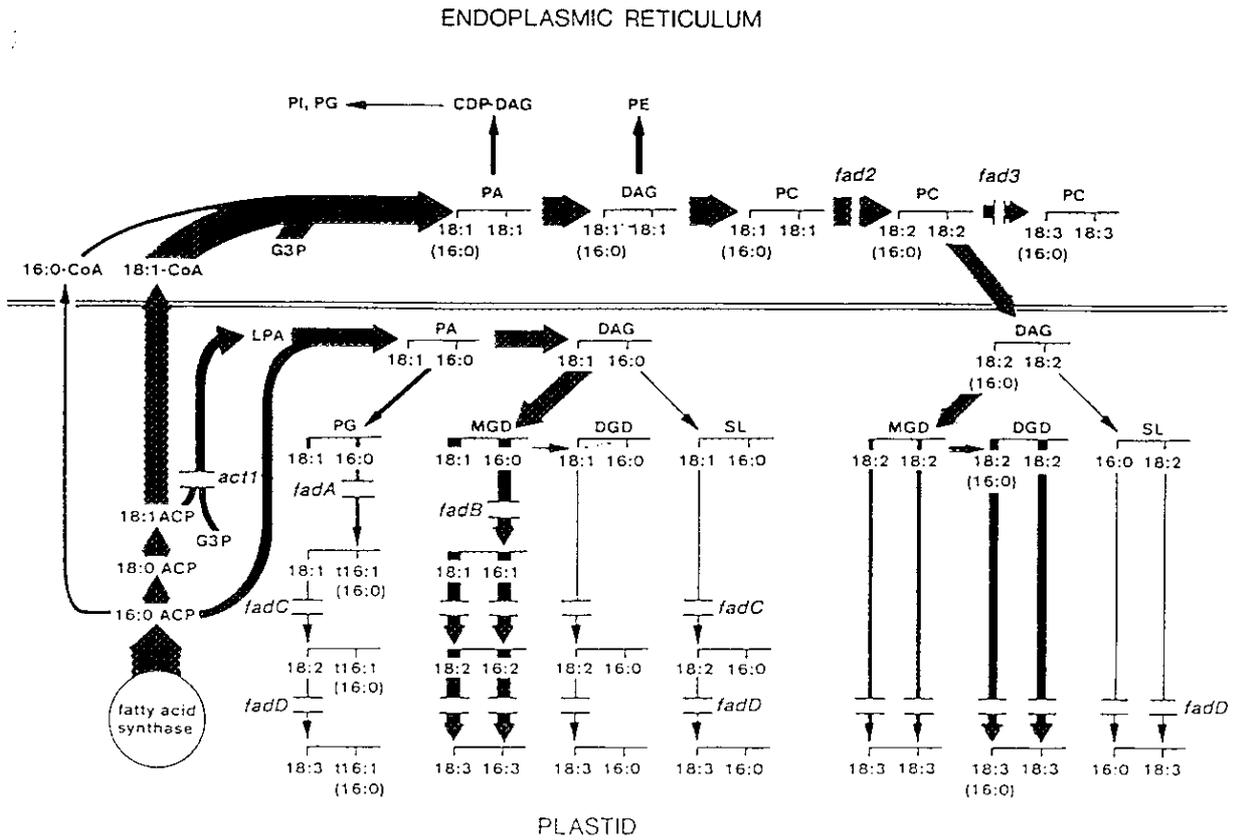


Figure 1-4. Mutants in lipid biosynthesis of *Arabidopsis* (Somerville and Browse 1991).

16:2(7,10) to 18:3(9,12,15) and 16:3(7,10,13) (Browse *et al.* 1986a). The *fad2* and *fad3* mutations deplete the desaturation of the $\Delta 12$ and $\Delta 15$ positions, respectively, of C₁₈ acids bound to PC in the endoplasmic reticula (Miquel and Browse 1992, Browse *et al.* 1993).

Biochemical and molecular genetical studies of fatty acid desaturases in plants

(1) Acyl-ACP desaturases

The $\Delta 9$ 18:0-ACP desaturase (EC. 1.14.99.6) catalyzes the conversion of 18:0-ACP to 18:1(9)-ACP in the presence of molecular oxygen, NADPH, NADPH:ferredoxin oxidoreductase and ferredoxin (Nagai and Bloch 1968). The enzyme has been extensively studied, because it is a water-soluble protein in the plastid. The $\Delta 9$ 18:0-ACP desaturase of maturing seeds of safflower is a homodimer with a molecular weight of 68,000 (McKeon and Stumpf 1982). The desaturases have been purified to homogeneity from avocado mesocarp (Shanklin and Somerville 1991) and developing embryos of safflower seed (Thompson *et al.* 1991). The cDNAs encoding the $\Delta 9$ 18:0-ACP desaturase were isolated from castor bean using monospecific polyclonal antibodies against the purified avocado desaturase (Shanklin and Somerville 1991), and from safflower based on the amino acid sequences of the purified protein (Thompson *et al.* 1991). At present, the cDNAs for the enzyme have been cloned from several plants: castor bean (Knutzon *et al.* 1991), cucumber (Shanklin *et al.* 1991), spinach (Nishida *et al.* 1992), *Brassica rapa* (Knutzon *et al.*

1992) and *Brassica napus* (Slocombe et al. 1992). The cDNA encoding the $\Delta 6$ 18:0-ACP desaturase was isolated from coriander (Cahoon et al. 1992) using the antibodies against the 18:0-ACP desaturase of avocado.

Gene-technological alterations of plant lipids were performed using the cDNAs of 18:0-ACP desaturases. Seed-specific antisense gene constructs of the $\Delta 9$ 18:0-ACP desaturase of *B. rapa* were used to reduce the enzyme activity in developing embryos during storage lipid biosynthesis (Knutzon et al. 1992). The resultant transgenic plants showed a marked decrease in the level of 18:1(9) associated with a increase in the level of 18:0. The transgenic tobacco callus, which was expressed with the $\Delta 6$ 18:0-ACP desaturase of coriander, accumulates 18:1(6) and 16:1(4), both of which were absent from control callus (Cahoon et al. 1992).

Recently, Fox et al. (1993) over produced the 18:0-ACP desaturase of castor bean in *E. coli* cells in catalytically active form. They demonstrated that the enzyme contains four atoms of iron per homodimer.

(2) Acyl-lipid desaturases

Plants have the other type of desaturases, which catalyze the desaturation of membrane-bound fatty acids. These desaturases are named "acyl-lipid desaturases". From a biochemical point of view, these enzymes are tightly integrated in membranes.

Schmidt and Heinz (1990a) succeeded in preparing a membrane fraction from detergent-treated chloroplasts that was active in desaturation of lipid-bound fatty acids. The membrane fraction was capable of desaturating 18:1(9) to 18:3(9,12,15), and a small extent of 16:0 to 16:3(7,10,13). The desaturation of lipid-bound fatty acids resembles that of 18:0-ACP with respect to its requirement of reduced ferredoxin and molecular oxygen. They also demonstrated that envelope membranes were able to desaturate 18:1(9) to 18:2(9,12) in MGDG-bound form (Schmidt and Heinz 1990b).

Molecular genetical approach to the desaturase genes has been succeeded in the ω 3 desaturases in the endoplasmic reticulum and plastid. Adrondel *et al.* (1992) isolated the cDNA encoding the cytoplasmic ω 3 desaturase of *Brassica napus*. They identified a clone of the yeast artificial chromosome (YAC) covering the *fad3* locus of *Arabidopsis thaliana* on the bases of the genetic map position. Then, they isolated a cDNA clone of *B. napus* which cross-hybridized with the YAC clone of *A. thaliana*. Expression of the cDNA of *B. napus* complemented the *fad3* mutation of *A. thaliana*. On the other hand, one of the auxin-regulated genes of mung bean, *ARG1* (Yamamoto *et al.* 1992), shows significant similarities to the *fad3* cDNA in the deduced amino-acid sequences, and is now regarded as the gene for the cytoplasmic ω 3 desaturase (Yamamoto 1993). Recently, Yadav *et al.* (1993) isolated the ω 3 desaturase gene (*fad3*) from *Arabidopsis thaliana* by T-DNA tagging. They also isolated cDNAs from *Arabidopsis*, soybean and rapeseed that

encode homologs of the cytoplasmic ω 3 desaturases (*fad3*) and also putative plastid ω 3 desaturases (*fadD*). Iba et al. (1993) isolated a gene encoding a chloroplast ω 3 desaturase (*fadD*) from *A. thaliana*. In addition, Okuley et al. (1994) isolated a *FAD2* gene encoding a Δ 12 desaturase of the endoplasmic reticulum from *A. thaliana*.

Roles of unsaturated fatty acids in higher plants

Roles of desaturation of membrane lipids have been studied with respect of adaptation of plants to changes of growth temperature. It has been demonstrated (Murata 1983) that the chilling sensitivity of plants is correlated with the degree of unsaturation of fatty acids in PG of chloroplast membranes. The substrate specificity of the glycerol-3-phosphate acyltransferase in chloroplasts determines the proportion of the unsaturated fatty acids incorporated into the *sn*-1 position of glycerolipids (Murata 1983). The acyltransferase from chilling tolerant plants selects 18:1(9) from the mixture of 18:1(9) and 16:0 (Frentzen et al. 1983). By contrast, the enzyme from chilling sensitive plants accepts 18:1(9) and 16:0 from the acyl-ACP pool (Frentzen et al. 1987). Murata et al. (1992a) demonstrated that the chilling tolerance of higher plants can be altered by genetic manipulation of the substrate specificity of the glycerol-3-phosphate acyltransferase in chloroplasts. The transgenic plant of *Nicotiana tabacum*, in which the glycerol-3-phosphate acyltransferase of a chilling-resistant

plant, *Arabidopsis*, was overexpressed, acquired the tolerance of the photosynthetic activity to low temperature. On the other hand, the transformant with the expressed enzyme of a chilling-sensitive plant, squash, increased the chilling sensitivity. These observations were confirmed by the results of the transgenic *Arabidopsis* with the gene (*plsB*) encoding glycerol-3-phosphate acyltransferase of *E. coli* (Wolter et al. 1992). The overexpression of the *E. coli* enzyme increased the incorporation of 16:0 into the sn-1 position of PG and rendered the plant to be less tolerant to chilling.

Growth characteristic of the *fad2* mutants of *Arabidopsis* has been reported by Miquel et al. (1993). The *fad2* mutants, which are deficient in the $\Delta 12$ desaturases in endoplasmic reticula, could grow at 22°C as the wild-type plant. By contrast, at 12°C, the mutants failed to undergo stem elongation during reproductive growth, although they produced normal flowers and fertile seeds. Upon low-temperature treatment at 6°C, rosette leaves of the mutants gradually died, and the plants were inviable. These results demonstrate a requirement of substantial levels of membrane polyunsaturation for low temperature survival of plants.

The *fadD* mutants of *Arabidopsis* are deficient in the $\omega 3$ desaturase in the chloroplasts (Browse et al. 1986a). The *fadD* mutation was suppressed when the *fadD* mutants were grown at low temperature, for example 18°C. The fatty acid composition of the *fadD* mutants grown at low temperature was similar to that of the wild-type plants. It is unlikely

that this effect is due to a temperature sensitive mutation, because several mutations of the *fadD* locus showed the same properties. There was no apparent effect of growth temperature on the levels of *fadD* transcription in wild-type plants (Iba et al. 1993). The suppression of the mutant phenotype the *fadD* mutants at low temperature implies induction or activation of another ω 3 desaturase in chloroplasts (Ohlrogge et al. 1991).

The *fadD* mutants do not show any obvious phenotype, demonstrating in a general sense that high concentrations of trienoic acids are not required for growth and development of plants under normal conditions (McCourt et al. 1987). The most pronounced change in the mutants was a 45% reduction in the cross sectional area of chloroplasts, associated with an increase in chloroplast number. These observations imply that the high trienoic acid content of thylakoid membranes may be related to chloroplast morphogenesis rather than directly to photosynthesis (McCourt et al. 1987). Recently, Iba et al. (1993) demonstrated that expression of the *fadD* cDNA in mutant plants resulted in restoration of wild type fatty acid composition and suppression of a effect of the *fadD* mutation on chloroplast number.

Desaturation of fatty acids in other organisms

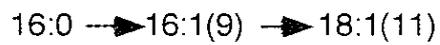
(1) Mammal

In animals, fatty acids of dietary or endogenous origin are modified by a combination of desaturation and chain elongation to produce longer-chain polyunsaturated fatty acids (Holloway 1983). The four major biosynthetic pathways are shown in Fig. 1-5. In animals the double bonds can be introduced at the $\Delta 4$, $\Delta 5$, $\Delta 6$, $\Delta 8$ and $\Delta 9$ positions (but never beyond the $\Delta 9$ position), and are introduced as *cis* configurations.

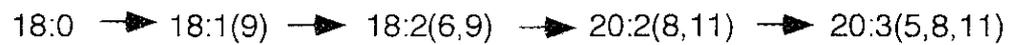
The $\Delta 9$ desaturation system, which catalyzes the conversion of 18:0-CoA to 18:1(9)-CoA, has been extensively studied using liver microsomes. This system is composed of three components, i.e., cytochrome *b*₅, cytochrome *b*₅ reductase and a terminal desaturase (Holloway 1971), and requires molecular oxygen and NADH (Marsh and James 1962). The terminal desaturases (18:0-CoA desaturases) was purified to homogeneity from rat liver microsomes (Strittmatter *et al.* 1974) and from chicken liver microsomes (Prasad and Joshi 1979).

The enzyme purified from rat liver microsomes is a single polypeptide of 53 kDa, containing one atom of non-heme iron (Strittmatter *et al.* 1974). The modification of arginyl residues by 2,3-butanedione and 1,2-cyclohexanedione inactivated the 18:0-CoA desaturase. The butanedione-treated enzyme can be reactivated by the removal of the reagent, and the recovery of activity is correlated with the regeneration of arginyl residues. The substrate, 18:0-CoA, protected the desaturase from inactivation by the chemical modifications. These data suggest that arginyl residues may

(1) Palmitic acid series



(2) Oleic acid series



(3) Linoleic acid series

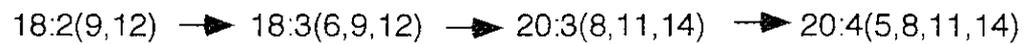
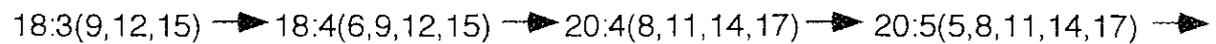
(4) α -linolenic acid series

Figure 1-5. Four major biosynthetic pathways of unsaturated fatty acids in animals (Holloway 1983).

play a role at the binding site of the substrate (Enoch and Strittmatter 1978). The desaturase is also inactivated by chemical modifications of tyrosyl residues: nitration by tetranitromethane and *O*-acetylation by *N*-acetylimidazole or acetic anhydride. Both nitration and acetylation of the desaturase caused a loss of the iron prosthetic group from the protein. This suggests that tyrosyl residues may be involved in binding of the non-heme iron (Enoch and Strittmatter 1978).

The 18:0-CoA desaturase purified from chicken liver has a molecular weight of 33 kDa, which is considerably smaller than that of the rat liver enzyme (Prasad and Joshi 1979). The antibody raised against the chicken liver enzyme did not cross-react with the purified enzyme of rat liver. The chicken enzyme is inhibited by both cyanide and azide, suggesting the involvement of non-heme iron in catalysis. In addition, the enzyme is completely inhibited by *p*-chloromercuribenzenesulfonate and this inhibition can be prevented by 18:0-CoA, suggesting the involvement of a sulfhydryl group for enzyme activity (Prasad and Joshi 1979). Topology of the 18:0-CoA desaturase of chicken was also studied by Prasad *et al.* (1980). The anti-desaturase antibody inhibited the terminal desaturase activity in the native microsomes. The terminal desaturase activity and its antigenic determinants in microsomal vesicles were susceptible to trypsin digestion. These data suggest that the chicken desaturase is localized on the cytoplasmic surface of the endoplasmic reticulum and that the active

site of the desaturase is exposed to the cytoplasm, as in the case of cytochrome *b*₅ and cytochrome *b*₅ reductase.

The Δ6 desaturase, which catalyzes the conversion of 18:2(9,12) to 18:3(6,9,12), has been purified from rat liver microsomes by Okayasu *et al.* (1981). The purified enzyme is a single polypeptide of 66 kDa containing one atom of non-heme iron. The Δ6 desaturation of 18:2(9,12)-CoA requires NADH, molecular oxygen, lipid or detergent, and three enzymes, *i.e.*, cytochrome *b*₅, cytochrome *b*₅ reductase and the terminal desaturase. These properties are similar to those of 18:0-CoA desaturases.

The Δ5 desaturase, which desaturates 20:3(8,11,14) to 20:4(5,8,11,14) (arachidonic acid) has been partially purified from rat liver microsomes (Pugh and Kates 1979). This system also requires molecular oxygen, NADH, cytochrome *b*₅, cytochrome *b*₅ reductase and the terminal desaturase.

The cDNA for rat liver 18:0-CoA desaturase has been isolated by Thiede *et al.* (1986). The cDNA includes a 1074-base open reading frame coding for 358 amino acids, corresponding a molecular mass of 41.1 kDa. In addition, the genomic DNA and cDNA clones for 18:0-CoA desaturase have been isolated from the mouse 3T3-L1 adipocytes (Ntambi *et al.* 1988). The deduced amino-acid sequence (355 amino-acid residues) of the mouse 3T3-L1 adipocyte 18:0-CoA desaturase exhibits 92% identity to that of rat liver 18:0-CoA desaturase. They also studied expression of the 18:0-CoA desaturase gene during differentiation of the mouse adipocyte (Ntambi *et al.* 1988). A chimeric construct,

containing 5'-flanking sequence of the mouse 18:0-CoA desaturase gene joined to the bacterial chloramphenicol acetyltransferase gene, was transfected into 3T3-L1 cells. When cells were induced to differentiate into adipocytes, expression of the chloramphenicol acetyltransferase activity increased 63-fold, suggesting that the promoter region of 18:0-CoA desaturase gene contains elements that mediate the response to adipogenic agents which induce differentiation.

Although animals cannot synthesize 18:2(9,12) *de novo*, *Tetrahymena pyriformis* (Kameyama *et al.* 1980), *Acheta domesticus* (cricket) (Cripps *et al.* 1990) and *Acanthamoeba castellanii* (Johnes *et al.* 1993) are exceptional in this respect. The activity of microsomal $\Delta 12$ desaturase was observed in these organisms. These $\Delta 12$ desaturases of *T. pyriformis* and *A. castellanii* were increased after the downward shift of growth temperature (Kameyama *et al.* 1980, Johnes *et al.* 1993).

(2) Fungi

In the desaturation system of *Saccharomyces cerevisiae*, 16:0 and 18:0 are desaturated to 16:1(9) and 18:1(9), respectively, in the form of CoA derivatives. Cytochrome *b₅* is involved in the desaturation system of yeast microsomes (Ohba *et al.* 1979).

Strains of *S. cerevisiae* bearing the *ole1* mutation are defective in synthesis of unsaturated fatty acids and require unsaturated fatty acids for growth (Resnick and Mortimer 1966). It had been assumed that *OLE1* was the

structural gene encoding the terminal enzyme of the $\Delta 9$ desaturation system for a long time. Stukey et al. (1990) isolated and characterized the *OLE1* gene of *S. cerevisiae*. The *OLE1* structural gene encodes a protein of 510 amino acids having a molecular mass of 57.4 kDa. A 257-amino acid internal region of the deduced amino-acid sequence of the *OLE1* gene shows 36% identity and 60% similarity to that of the rat liver 18:0-CoA desaturase. The expression of the *OLE1* gene is repressed by monounsaturated and polyunsaturated fatty acids containing a double bond at the $\Delta 9$ position added to the growth medium (McDonough et al 1992).

Wilson et al. (1980) reported the $\Delta 12$ desaturation of 18:1(9)-CoA by the microsomal fraction of *Fusarium oxysporum* hyphal cells. This reaction required molecular oxygen and NADPH. Direct desaturation of phospholipids, apart from that of acyl-CoA derivatives, has been reported in yeasts, *Candida lipolytica* (Pugh and Kates 1973, 1975) and *Torulopsis utilis* (Talamo et al. 1973). The microsomal phospholipid desaturase of *C. lipolytica* catalyzes the desaturation of 18:1(9) to 18:2(9,12) bound to both the *sn*-1 and *sn*-2 positions of PC. The reaction required molecular oxygen and cytochrome *b*₅ (Pugh and Kate 1975). Talamo et al. (1973) demonstrated that cell-free extract from *T. utilis* contained the activities of the desaturation of both 1,2-dioleoyl PC and 18:1-CoA to form 18:2(9,12). These phospholipid desaturases of yeasts have not been purified to homogeneity.

(2) Bacteria

In bacterial systems, unsaturated fatty acids are synthesized via 'the anaerobic pathway', which is extensively studied in *Escherichia coli* (Bloch 1971). Bacteria generally introduce a *cis* double bond during fatty acid biosynthesis. A specific β -hydroxydecanoyl-ACP dehydrase catalyzes the dehydration of β -hydroxydecanoyl-ACP to *cis*- β,γ -decanoyl-ACP. The *cis*- β,γ -decanoyl-ACP subsequently elongate to 16:1(9) and 18:1(11) by the common reactions of fatty acid synthesis. Owing to the association with the dehydrase, such bacterial fatty acid synthetases catalyze a branched pathway leading to unsaturated fatty acids as well as saturated fatty acids (Fig. 1-6).

There are exceptions to the generalization; the aerobic desaturation system found in certain bacteria (Fulco 1974). The $\Delta 9$ desaturation system has been reported in *Mycobacterium phlei* (Fulco and Bloch 1964, Kashiwabara and Sato 1973). Cell-free extracts of *M. phlei* catalyzed the conversion of 18:0-CoA and 16:0-CoA to the $\Delta 9$ -unsaturated derivatives in the presence of a flavin (FAD or FMN), Fe^{2+} , NADH and molecular oxygen. *Bacillus megaterium* desaturates 16:0 and 18:0 to form the *cis*- $\Delta 5$ derivatives by oxygen-dependent pathway (Fulco et al. 1964). The $\Delta 5$ desaturase in *B. megaterium* is transiently synthesized after a shift of growth temperature from 35°C to 20°C (Fujii and Fulco 1977). Dart and Kaneda (1970) reported the $\Delta 10$ desaturation system

in *Bacillus cereus*. In *Vibrio* sp. strain ABE-1, the anaerobic pathway and aerobic desaturation are both involved in the synthesis of unsaturated fatty acids (Morita et al. 1992). 14:1(7) is synthesized by the anaerobic pathway, while 16:1 is synthesized by the aerobic desaturation of 16:0, during aerobic growth of *Vibrio*.

The electron transfer mechanism involved in these bacterial desaturation system remains unclear. These desaturases of bacteria have not been purified to homogeneity.

Aim of the present study

Polar glycerolipids are major constituents of biological membranes. The physical properties of biological membranes depend on the fatty acid composition of membrane lipids; the degree of unsaturation and the chain length of the fatty acids affects the thermotropic phase behavior of membranes. Cyanobacteria respond to a decrease in ambient temperature by desaturating fatty acids of membrane lipids to compensate the decrease in membrane fluidity at low temperature.

The fatty acid desaturases are the enzymes that introduce double bonds into hydrocarbon chain of fatty acids. The structural information of desaturases is necessary to understand the reaction mechanism of the fatty acid desaturation. In order to solve the role of unsaturation of membrane lipids, it is required to control the degree of membrane lipid unsaturation by genetic

manipulation of the fatty acid desaturase genes. In addition, characterization of the temperature-dependent regulation of desaturase genes in cyanobacteria contributes toward understanding the molecular mechanism of temperature acclimation of cyanobacteria.

The aims of the present study are: (1) Isolation and characterization of the cyanobacterial desaturase genes; (2) Alteration of unsaturation of membrane lipid in cyanobacteria by the manipulation of the isolated desaturase genes.

Chapter 2

Identification of the conserved domains of the $\Delta 12$
desaturases of cyanobacteria.

Abstract

Cyanobacterial genes for enzymes that desaturate fatty acids at the $\Delta 12$ position, designated *desA*, were isolated from *Synechocystis* PCC6714, *Synechococcus* PCC7002 and *Anabaena variabilis* by cross-hybridization with a DNA probe derived from the *desA* gene of *Synechocystis* PCC6803. The genes of *Synechocystis* PCC6714, *Synechococcus* PCC7002 and *A. variabilis* encode proteins of 349, 347 and 350 amino acid residues, respectively. The transformation of *Synechococcus* PCC7942 with the *desA* genes from *Synechocystis* PCC6714, *Synechococcus* PCC7002 and *A. variabilis* was associated with the ability to introduce a second double bond at the $\Delta 12$ position of fatty acids. The amino acid sequence of the products of the *desA* genes revealed the presence of four conserved domains. Since one of the conserved domains was also found in the amino acid sequences of $\omega 3$ desaturases of *Brassica napus* and mung bean, this domain may play an essential role in the introduction of a double bond into fatty acids bound to membrane lipids.

Introduction

In cyanobacteria, the unsaturated fatty acids of membrane lipids are synthesized by introduction of double bonds into saturated fatty acids that are bound to glycerolipids (Sato and Murata 1982a,b, Sato et al. 1986). Recently, Wada et al. (1993) demonstrated that components involved in this reaction are ferredoxin, ferredoxin:NADP oxidoreductase, and the terminal desaturase, as is also the case in higher plants (Schmidt and Heinz 1990a). The introduction of double bonds into fatty acids that are bound to glycerolipids is a unique property of the desaturases of plants and cyanobacteria (Jaworski 1987). Accordingly, these desaturases are named "acyl-lipid desaturases".

Cyanobacteria can be classified into four groups with respect to the mode of desaturation of fatty acids (Murata et al. 1992b). Strains in the first group can desaturate only at the $\Delta 9$ position. The second, third, fourth groups are characterized by the ability to introduce double bonds at the $\Delta 9$, $\Delta 12$ and $\omega 3$ positions, at the $\Delta 6$, $\Delta 9$ and $\Delta 12$ positions and at the $\Delta 6$, $\Delta 9$, $\Delta 12$ and $\omega 3$ positions, respectively. Certain unicellular cyanobacteria, namely *Synechococcus* PCC7942, *Synechococcus* PCC7002, *Synechocystis* PCC6714, and *Synechocystis* PCC6803, are known to be amenable to transformation (Golden et al. 1987, Haselkorn 1991). These four strains belong, respectively, to the first above mentioned group, the second group, the third group, and the fourth group, respectively (Murata et al. 1992b). The

filamentous cyanobacterium *Anabaena variabilis* belongs to the second group (Murata *et al.* 1992b).

Wada *et al.* (1990) have cloned the gene for the $\Delta 12$ desaturase, *desA*, from *Synechocystis* PCC6803. Recently, a gene for the $\Delta 6$ desaturase was cloned from the same cyanobacterium (Reddy *et al.* 1993). cDNAs for $\omega 3$ desaturases have also been cloned from *Brassica napus* (Arondel *et al.* 1992) and mung bean (Yamamoto *et al.* 1992). The $\Delta 6$ desaturase shows limited similarity at the amino acid level to the $\Delta 12$ desaturase of *Synechocystis* PCC6803 and to the $\omega 3$ desaturases of *B. napus* and mung bean. However, there is no apparent similarity in terms of amino acid sequence between these desaturases and the 18:0-CoA desaturases isolated from rat (Thiede *et al.* 1986), mouse (Ntambi *et al.* 1988) and *Saccharomyces cerevisiae* (Stukey *et al.* 1990).

In order to identify the domains that are specific to the $\Delta 12$ desaturases, it is necessary to compare the *desA* genes from various sources. In the present study, we isolated the *desA* genes from *Synechocystis* PCC6714, *Synechococcus* PCC7002 and *A. variabilis* by cross-hybridization with the *desA* gene of *Synechocystis* PCC6803, and the author found four conserved domains in the amino acid sequences that were deduced from the nucleotide sequences of the various *desA* genes.

Material and methods

Organisms and culture conditions---*Synechocystis* PCC6714, *A. variabilis* and *Synechococcus* PCC7942 were grown photoautotrophically at 34°C in BG-11 medium (Stanier et al. 1971), supplemented with 20 mM HEPES-NaOH (pH 7.5), under illumination from incandescent lamps ($70 \mu\text{E m}^{-2} \text{s}^{-1}$) with aeration of 1.0% CO₂ in air as described previously (Wada and Murata 1989). *Synechococcus* PCC7002 was grown in the same way, but in medium A (Stevens et al. 1973). Transformants of *Synechococcus* PCC7942 were selected and maintained on BG-11 medium supplemented with 10 $\mu\text{g ml}^{-1}$ streptomycin for both liquid culture and growth on plates.

Construction of genomic libraries---Genomic DNA libraries of *Synechocystis* PCC6714, *Synechococcus* PCC7002 and *A. variabilis* were constructed using the phage vector $\lambda\text{DASH II}$ (Stratagene, La Jolla, CA, USA). Cyanobacterial genomic DNA was prepared as described by Williams (1988). The genomic DNA was partially digested with *Sau3A I* and integrated into the *BamH I* site of the vector.

Screening of the genomic libraries---The *desA* probes of 0.25 kbp (position 416 to position 661 in the nucleotide sequence deposited in the EMBL data bank with the accession number, X53508) and 1.1 kbp (position 187 to position 1233) were excised from the plasmid Bluescript/1.5-kbp that contained the *desA* gene of *Synechocystis* PCC6803 (Wada et al. 1990) by *EcoO109 I* digestion, and by *Hinc II* and *Spe I* digestion,

respectively. ^{32}P -Labeling of DNA fragments was performed with a random primer labeling kit (Takara, Kyoto, Japan). Plaque hybridization was performed essentially as described by Ausubel et al. (1987). Plaques were transferred onto nylon membranes (GeneScreen Plus; Du Pont/NEN Research Products, Boston, MA, USA), and hybridized with the probe at 50°C for 42 h in 0.09 M sodium citrate buffer (pH 7.0) that contained 0.9 M NaCl, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 10% dextran sulfate, 0.1% SDS and $100\ \mu\text{g ml}^{-1}$ salmon sperm DNA. After hybridization, the membranes were washed with 0.015 M sodium citrate buffer (pH 7.0) that contained 0.15 M NaCl and 0.5% SDS, at 50°C , and then they were exposed to X-ray film (WIF50; Konica, Tokyo, Japan).

Transformation of Synechococcus PCC7942---The 6-kbp DNA fragment from *Synechocystis* PCC6714, the 10-kbp DNA fragment from *Synechococcus* PCC7002 and the 7-kbp DNA fragment from *A. variabilis*, all containing a *desA* gene, were subcloned into the *EcoR* I site of an *Escherichia coli*-*Synechococcus* PCC7942 shuttle vector, pUC303 (Kuhlemeier et al. 1983). The resultant plasmids, designated pUC303/6-kbp, pUC303/10-kbp and pUC303/7-kbp, respectively, were used for the transformation of *Synechococcus* PCC7942, which has no detectable $\Delta 12$ desaturase activity (Murata et al. 1992b). The transformation was accomplished essentially as described by Williams and Szalay (1983). Streptomycin-resistant

transformants were selected on BG-11 plates that contained 10 $\mu\text{g ml}^{-1}$ streptomycin.

Analysis of fatty acids---Cells were collected by centrifugation and lyophilized. They were then subjected to methanolysis in 5% HCl (w/w) in methanol at 85°C for 2.5 h. The resultant methyl esters were extracted with *n*-hexane and analyzed by gas-liquid chromatography as described previously (Wada and Murata 1989). Fatty acid methyl esters were identified with a gas-liquid chromatograph and a gas chromatograph-mass spectrometer. The positions of double bonds were determined with a gas chromatograph-mass spectrometer as described previously (Wada and Murata 1989).

Determination of nucleotide sequences---The *Hind* III 2.7-kbp DNA fragment from *Synechocystis* PCC6714, the *Hinc* II 4.8-kbp DNA fragment from *Synechococcus* PCC7002, and the *Cla* I 3-kbp DNA fragment from *A. variabilis*, all containing a *desA* gene, were subcloned into pBluescript II KS(+) (Stratagene). Nested deletions were constructed using a Kilo-Sequence Deletion Kit (Takara). The nucleotide sequences were determined by the dideoxy chain-termination method (Sanger et al. 1977) using a 7-deaza dGTP DNA sequencing kit (Takara) and a BcaBEST sequence kit (Takara). The entire sequence of DNA was determined in both directions. The multiple alignment of the deduced amino-acid sequences was performed using a system for molecular evolutionary analysis of DNA and amino acid sequences, ODN, at the National Institute of Genetics.

Results

Screening of genomic DNA libraries---The 0.25-kbp DNA fragment of the coding region of the *desA* gene of *Synechocystis* PCC6803 was used for screening of the genomic libraries of *Synechocystis* PCC6714 and *Synechococcus* PCC7002. Thirty-nine positive clones were obtained from 5×10^3 recombinants in the genomic library of *Synechocystis* PCC6714. The author selected one of the positive clones, which produced fragments of 2.7 kbp and 6 kbp, respectively, with *Hind* III and *EcoR* I digestion, that hybridized with the screening probe. The 6-kbp *EcoR* I fragment was subcloned into the *EcoR* I site of pUC303, and the resultant plasmid was designated pUC303/6-kbp. Nine positive clones were obtained from 3.5×10^3 recombinants in the *Synechococcus* PCC7002 genomic library. The author selected one of the positive clones, which produced fragments of 4.8 kbp and 10 kbp, respectively, with *Hinc* II and *EcoR* I digestion, that hybridized with the screening probe. The 10-kbp *EcoR* I fragment was subcloned into the *EcoR* I site of pUC303, and the resultant plasmid was designated pUC303/10-kbp.

The 1.1-kbp DNA fragment of the *desA* gene of *Synechocystis* PCC6803 was used for screening of the genomic library of *A. variabilis*. Four positive clones were obtained from 3.5×10^3 recombinants in the genomic library of *A. variabilis*. The author selected one of the positive clones, which produced fragments of 2.1 kbp and 7 kbp, respectively, with *Hinc* II and *EcoR* I digestion, that hybridized with the

screening probe. The 7-kbp *EcoR* I fragment was subcloned into the *EcoR* I site of pUC303, and the resultant plasmid was designated pUC303/7-kbp.

Fatty acid compositions of transformants---Synechococcus PCC7942 was transformed with the vector plasmids, pUC303/6-kbp, pUC303/10-kbp and pUC303/7-kbp, which contained fragment of DNA from *Synechocystis* PCC6714, *Synechococcus* PCC7002, and *A. variabilis*, respectively. The fatty acid compositions of total lipids isolated from wild-type *Synechococcus* PCC7942 and the transformants are shown in Table 2-1. The wild-type cells contained saturated and monounsaturated fatty acids, such as 16:0, 16:1(9) and 18:1(9), but no polyunsaturated fatty acids. In cells transformed with the unmodified vector plasmid, the fatty acid composition was the same as that of wild type. However, cells transformed with either pUC303/6-kbp or pUC303/10-kbp contained 16:2(9,12) and 18:2(9,12) in addition to saturated and monounsaturated fatty acids. Cells transformed with pUC303/7-kbp contained 18:2(9,12). Although levels of the fatty acids with the Δ 12 unsaturation were variable among the transformants, their presence was clearly demonstrated. These results indicate that *Synechococcus* PCC7942 acquired Δ 12 desaturase activity as a consequence of the introduction of the specific fragments of DNA from *Synechocystis* PCC6714, *Synechococcus* PCC7002 and *A. variabilis*, and that the isolated fragments of DNA included genes responsible for desaturation of fatty acids at the Δ 12 position.

Table 2-1. Fatty acid compositions of total lipids from wild-type *Synechococcus* PCC7942 and transformants

Strain	Fatty acid								
	14:0	16:0	16:1 (9)	16:2 (9,12)	18:0	18:1 (11)	18:1 (9)	18:2 (9,12)	18:2 ^a
(mol %)									
Wild type	t	50	34	0	4	5	6	0	0
Transformant									
with pUC303	t	51	37	0	3	3	5	0	0
with pUC303/6-kbp from 6714	0	54	26	12	2	1	1	3	1
with pUC303/10-kbp from 7002	t	54	29	5	3	4	1	3	1
with pUC303/7-kbp from <i>Anabaena</i>	1	55	34	0	2	3	5	1	0

t: Trace (less than 0.5%).

^a The positions of double bonds were not determined.

The values represent averages of results from three independent measurements.

Nucleotide sequences---A *Hind* III 2.7-kbp fragment of the 6-kbp fragment from *Synechocystis* PCC6714 was subcloned into the *Hind* III site of pBluescript II KS(+). The nucleotide sequence of a 1.2-kbp region of the subcloned fragment, which hybridized with the probe, was determined. The nucleotide sequence of 1176 bp is represented in Appendix A. An open-reading frame of 1047 nucleotides was found. It corresponded to a polypeptide of 349 amino acids with an approximate molecular mass of 40.3 kDa.

A *Hinc* II 4.8-kbp fragment of the 10-kbp fragment from *Synechococcus* PCC7002 was subcloned into the *Hinc* II site of pBluescript II KS(+), and the nucleotide sequence of a 1.3-kbp region of the subcloned fragment, which hybridized with the probe, was determined. The nucleotide sequence of 1251 bp is represented in Appendix B. An open-reading frame of 1041 nucleotides was found, corresponding to a polypeptide of 347 amino acids with an approximate molecular mass of 40.7 kDa.

A *Cla* I 3-kbp fragment of the 7-kbp fragment from *A. variabilis* was subcloned into the *Cla* I site of pBluescript II KS(+), and the nucleotide sequence of a 2.6-kbp region that hybridized with the probe, was determined. The nucleotide sequence of 2618 bp is represented in Appendix C. An open-reading frame of 1050 nucleotides was found, corresponding to a polypeptide of 350 amino acids with an approximate molecular mass of 41.3 kDa.

Discussion

The amino acid sequences deduced from the nucleotide sequences of the *desA* genes are compared in Fig. 2-1. The product of the *desA* gene of *Synechocystis* PCC6714 is similar to that of *Synechocystis* PCC6803. The score for the sequence similarity between the products of the *desA* genes of *Synechocystis* PCC6714 and *Synechocystis* PCC6803 is 96%. However, the scores for the sequence similarities between the *desA* products of *Synechococcus* PCC7002 and *Synechocystis* PCC6803 and between the *desA* products of *A. variabilis* and *Synechocystis* PCC6803 are 57% and 59%, respectively. A comparison of the amino acid sequences indicates the presence of four conserved domains. (Fig. 2-1 boxed)

Figure 2-2 compares hydropathy profiles of the products of the *desA* genes of *Synechocystis* PCC6803, *Synechocystis* PCC6714, *Synechococcus* PCC7002, and *A. variabilis*. They are very similar to one another, each having two hydrophobic regions, which are putative membrane-spanning domains. The four conserved domains mentioned above are located in regions outside the hydrophobic regions.

The consecutive amino-acid residues: -G-H-D-C-G-H-, (Fig. 2-1, underlining) are conserved between the $\Delta 12$ desaturases of cyanobacteria and the $\omega 3$ desaturases of higher plants (Arondel *et al.* 1992, Yamamoto *et al.* 1992). This arrangement of amino acids is found in the first conserved domain of the $\Delta 12$ desaturases. Thus, the first conserved domain must play an essential and common role in the

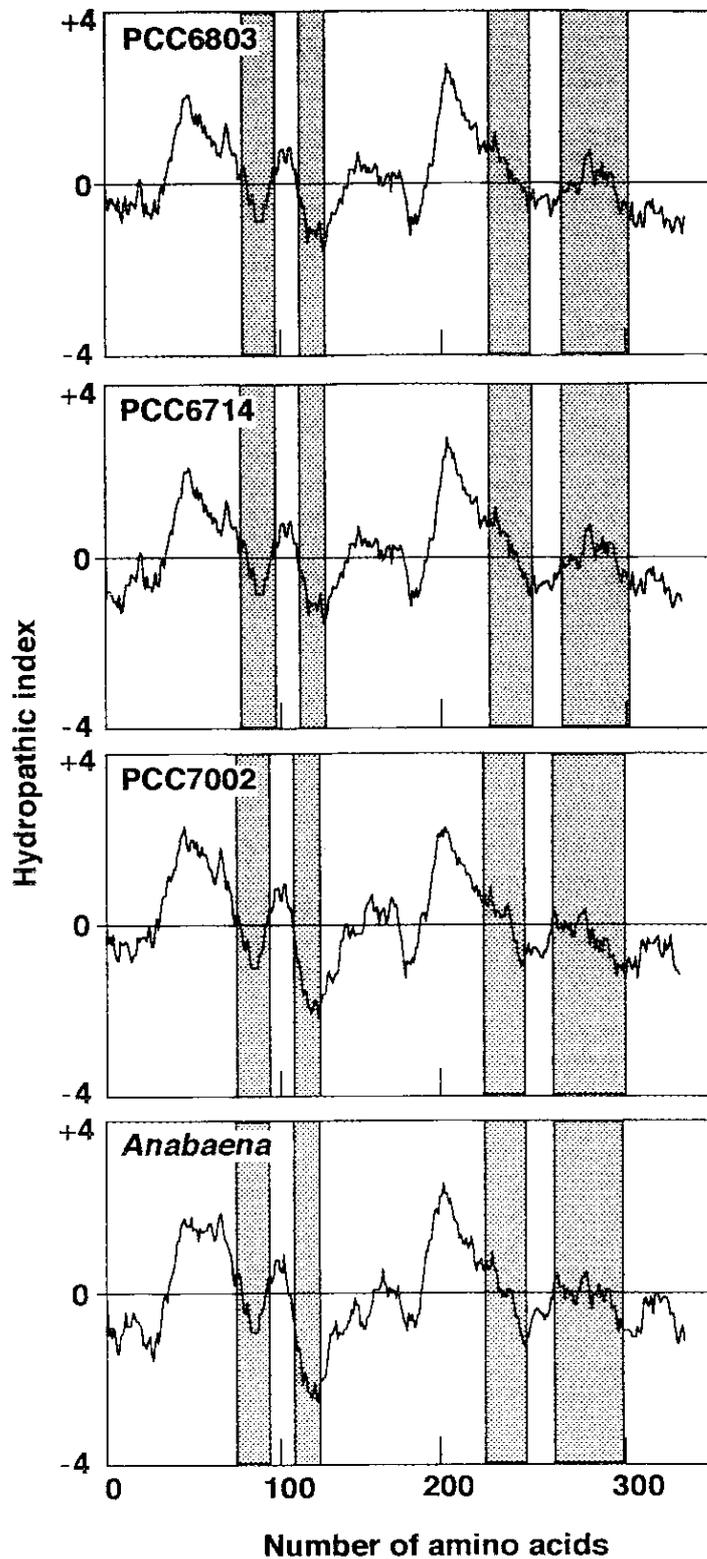


Figure 2-2. Hydropathy profiles of the products of the *desA* genes from *Synechocystis* PCC6803, *Synechocystis* PCC6714, *Synechococcus* PCC7002 and *Anabaena variabilis*. Hydropathy profile was calculated by the method of Kyte and Doolittle (1982) with a window size of 19 amino-acid residues. The conserved domains in the $\Delta 12$ desaturases are indicated by shadowing.

desaturation reaction acting, for example, as the catalytic center, as the site for binding or recognition of the fatty acids, or as the site of an electron acceptor. The other conserved domains in the $\Delta 12$ desaturases may determine the position of the double bond to be introduced into the fatty acids.

When the *desA* genes from the various cyanobacteria were introduced into *Synechococcus* PCC7942 cells, they were expressed and their products were active in desaturation at the $\Delta 12$ position. This result indicates that the electron-donating system in *Synechococcus* PCC7942 can operate to support the function of these desaturases regardless their origin. Since the cells transformed with the DNA fragment from *A. variabilis* synthesized the fatty acids of the $\Delta 12$ unsaturation at low levels, it is likely that the $\Delta 12$ desaturase of *A. variabilis* was not well expressed in *Synechococcus* PCC7942, or that the desaturase of *A. variabilis* does not well react with ferredoxin of *Synechococcus* PCC7942. By contrast, the $\Delta 12$ desaturases of *Synechocystis* PCC6714 and *Synechococcus* PCC7002 can catalyze the desaturation of both 16:1(9) and 18:1(9). It remains open to question whether the $\Delta 12$ desaturases recognize acyl groups at the *sn*-1 and *sn*-2 positions or the polar head group at the *sn*-3 position of glycerol moiety as their substrates. Structural information about the $\Delta 12$ desaturases will provide clues for the analysis of the molecular mechanism of desaturation of fatty acids and the mechanism responsible for recognition of double bonds by the desaturases.

Chapter 3

The genetic manipulation of membrane lipid unsaturation with the ω 3 desaturase in cyanobacteria.

Abstract

Cyanobacteria respond to a decrease in temperature by desaturating the fatty acids of their membrane lipids to compensate for the decrease in membrane fluidity. Among the various desaturation reactions in cyanobacteria, the desaturation at the ω 3 position of fatty acids is the most sensitive to the change in temperature. In the present study, the author isolated a gene, designated *desB*, for the ω 3 desaturase of the cyanobacterium *Synechocystis* PCC6803, by cross-hybridization with a probe derived from the *desA* gene that encodes the Δ 12 desaturase. The *desB* gene encodes a protein of 359 amino-acid residues and of molecular mass of 41.9 kDa. In order to manipulate the fatty acid unsaturation of membrane lipids, the *desB* gene in *Synechocystis* PCC6803 was mutated by insertion of the kanamycin-resistance gene cartridge. The resultant mutant was unable to desaturate fatty acids at the ω 3 position. In another experiment, the *desA* previously cloned for the Δ 12 desaturase and the *desB* gene of *Synechocystis* PCC6803 were introduced into a different cyanobacterium *Synechococcus* PCC7942, which can desaturate membrane lipids only at the Δ 9 position of fatty acids. The resultant transformant was able to desaturate fatty acids of membrane lipids at the Δ 9, Δ 12 and ω 3 positions. These results demonstrate that disruption of the *desB* gene and its introduction into cells from which it is originally absent enables us to manipulate genetically the fatty acid unsaturation of membrane lipids in cyanobacteria.

Introduction

Most of cyanobacterial strains contain high levels of polyunsaturated fatty acids, as do higher plants (Murata and Nishida 1987). However, the biosynthesis of polyunsaturated fatty acids in cyanobacteria differs from that in higher plants. All reactions that result in the desaturation of fatty acids in cyanobacterial cells take place after the fatty acids have become bound to glycerolipids (Sato and Murata 1982a,b, Sato et al. 1986). In higher plants, the desaturation of 18:0 to 18:1(9) occurs when the fatty acid is in the ACP-bound form (McKeon and Stumpf 1982, Stumpf 1981), and all the other reactions that result in desaturation of fatty acids take place after the fatty acids have become bound to glycerolipids (Harwood 1988, Jaworski 1987). In higher plants, the enzyme that catalyzes the desaturation of 18:0-ACP, is a water-soluble protein which is localized in the stroma of chloroplasts. By contrast, the desaturases, which catalyze the desaturation of fatty acids bound to lipids, are membrane-bound (Slabas and Fawcett 1992). These membrane-bound desaturases in higher plants and cyanobacteria are called "acyl-lipid desaturases".

In the cyanobacterium *Synechocystis* PCC6803, polyunsaturated fatty acids are synthesized by sequential introduction of double bonds into C₁₈ fatty acids, which have been esterified to the *sn*-1 position of glycerolipids, by four distinct desaturases. Each of the enzymes catalyzes the desaturation of fatty acids at a specific position (Wada and

Murata 1989, 1990). A study of substrate specificity of the desaturases of *Synechocystis* PCC6803 that involved cells feeding with an odd-numbered aliphatic acid (Higashi and Murata 1993) demonstrated that each of the desaturases introduces a double bond at the $\Delta 6$, $\Delta 9$, $\Delta 12$ or $\omega 3$ position of fatty acids, respectively.

The $\Delta 12$ desaturase gene, *desA* (Wada et al. 1990), and the $\Delta 6$ desaturase gene (Reddy et al. 1993) have been cloned from *Synechocystis* PCC6803. A cDNA for the gene, *fad3*, that encodes the cytoplasmic desaturase that acts at the $\omega 3$ position has been isolated from *Brassica napus* (Arondel et al. 1992). One of the auxin-regulated genes of mung bean, *ARG1* (Yamamoto et al. 1992), shows significant similarities to the *fad3* cDNA and to the *desA* gene in terms of the amino acid sequences deduced from each, and it is now regarded as the gene for the $\omega 3$ desaturase in the cytoplasmic reticula.

The unicellular cyanobacteria *Synechocystis* PCC6803 and *Synechococcus* PCC7942 can be transformed by exogenously added DNA (Haselkorn 1991). This property of these cyanobacteria has enabled us to examine the effects of manipulation of the *desA* gene on the biological roles of the unsaturation of fatty acids of membrane lipids. Disruption of the *desA* gene in *Synechocystis* PCC6803 results in elimination of the desaturation of fatty acids at the $\Delta 12$ position and renders the cyanobacterium sensitive to low temperature (Wada et al. 1992, Gombos et al. 1993). Introduction of the *desA* gene into *Synechococcus* PCC7942 allowed this strain to desaturate

fatty acids at the $\Delta 12$ position and increased its tolerance to low temperature (Wada et al. 1990).

In the present study, the author describes the isolation of the *desB* gene of *Synechocystis* PCC6803, which is responsible for acyl-lipid desaturation at the $\omega 3$ position of fatty acids, by cross-hybridization with a probe derived from the *desA* gene. By genetic manipulation using the *desB* gene, the author has been able to create cyanobacterial strains with different levels of unsaturation of fatty acids in membrane lipids.

Materials and Methods

Organisms and culture conditions---*Synechocystis* PCC6803 and *Synechococcus* PCC7942 were grown photoautotrophically at 34°C or 22°C in BG-11 medium supplemented with 20 mM HEPES-NaOH (pH 7.5) under illumination provided by incandescent lamps at 70 $\mu\text{E m}^{-2} \text{s}^{-1}$ with aeration, as described previously (Wada and Murata 1989). Mutants of *Synechocystis* PCC6803 and transformants of *Synechococcus* PCC7942 were selected and maintained on BG-11 medium (Stanier et al. 1971) supplemented with kanamycin at 25 $\mu\text{g ml}^{-1}$ and streptomycin at 10 $\mu\text{g ml}^{-1}$ for both liquid culture and growth on plates.

desA- Δ mutant---The author created a mutant of *Synechocystis* PCC6803, designated *desA- Δ* , in which the *desA* gene in the genome was replaced by the Km^{r} cartridge (Beck et al. 1982) as follows. A genomic library of wild-type *Synechocystis* PCC6803, constructed with the phage vector λEMBL3 , was kindly

provided by Dr. T. Omata (Nagoya University), who obtained it from Dr. J. G. K. Williams (E. I. du Pont de Nemours and Company). A *desA* probe of 1.2 kbp (position 1 to position 1233 in the nucleotide sequence deposited in the EMBL data bank with the accession number, X53508) was prepared by digestion of Bluescript/1.5 kbp (Wada et al. 1990) with *EcoR* I and *Spe* I, and subsequent labeling of the resultant fragment with [α -³²P]dCTP using a random primer labeling kit (Takara, Kyoto, Japan). A λ EMBL3 clone with an insert of 14 kbp that contained the *desA* gene was obtained by screening the genomic library with the *desA* probe. The *EcoR* I fragment of 3.0 kbp, which corresponds to the region upstream of the *desA* gene, was obtained from the λ EMBL3 clone. This fragment was subcloned into the *EcoR* I site of the plasmid, pBluescript II KS(+) (Stratagene, La Jolla, CA, USA), and the resultant plasmid was designated pBluescript/E3. The *Spe* I fragment of 1.0 kbp, which corresponds to the region downstream of the *desA* gene, was obtained from the same λ EMBL3 clone. This fragment was subcloned into the *Spe* I site of pBluescript II KS(+), and the resultant plasmid was designated pBluescript/S1. The Km^r cartridge of 1.2 kbp was prepared from pUC4 KIXX (Pharmacia, Uppsala, Sweden) by digestion of *Sma* I. This cartridge was inserted into the *EcoR* V site of pBluescript/E3 and the resultant plasmid was designated pBluescript/E3/Km. The 4.2-kbp fragment containing the 3.0-kbp fragment and the 1.2-kbp Km^r cartridge was prepared by digesting the pBluescript/E3/Km^r with *Sma* I and *Sal* I and generation of blunt ends by T4 DNA polymerase.

The 4.2-kbp fragment was inserted into the *Hinc* II site of pBluescript/S1. The resultant plasmid, containing the construct illustrated in Fig. 3-1A2, was designated pBluescript/E3/Km^r/S1.

Wild-type cells of *Synechocystis* PCC6803 were transformed with the pBluescript/E3/Km/S1 by the method of Golden et al. (1987). The resultant transformant was designated *desA-Δ*. The genomic DNA of the *desA-Δ* mutant was analyzed by DNA/DNA hybridization with the Km^r probe of 1.2 kbp and the *desA* probe of 1.2 kbp under highly stringent conditions in Rapid Hybridization Buffer (Amersham, Buckinghamshire, UK) as recommended manufacture's protocol.

Construction and screening of the genomic library of the desA-Δ mutant---The genomic DNA of the *desA-Δ* mutant, extracted as described by Williams (1988), was partially digested with *Sau*3A I and integrated into the *Bam*H I site of the phage vector λDASH II (Stratagene). The resultant genomic library was screened by plaque hybridization essentially as described by Ausubel et al (1987), as follows. Approximately 3.5×10^3 plaques were transferred onto nylon membranes (GeneScreen Plus; Du Pont/NEN Research Products, Boston, MA, USA), and hybridized in 0.09 M sodium citrate buffer (pH 7.0) that contained 0.9 M NaCl, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 10% dextran sulfate, 0.1% SDS and $100 \mu\text{g ml}^{-1}$ salmon sperm DNA, at 50°C for 42 h with a probe of 0.25 kbp derived from the *desA* gene. This probe was prepared by digestion with *Eco*O109 I of

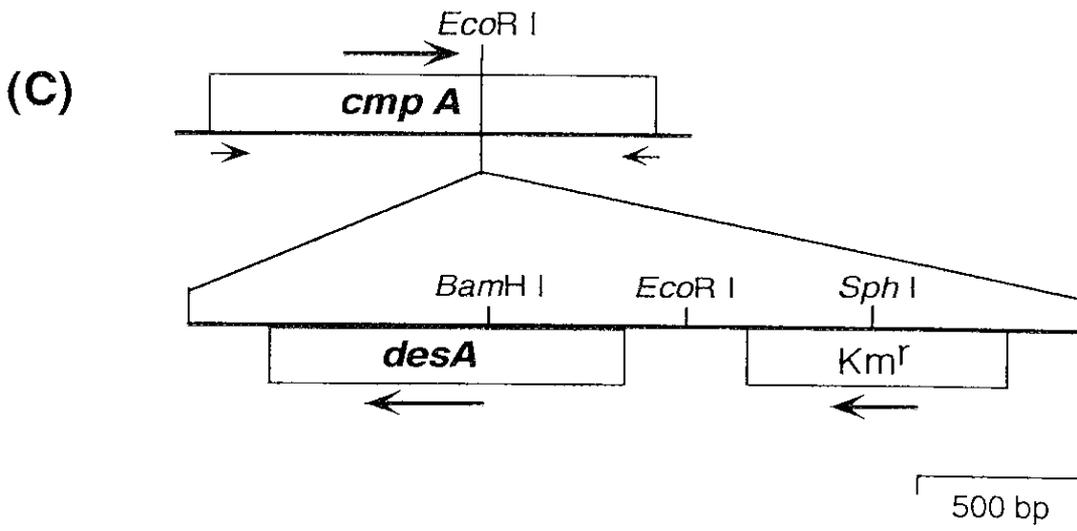
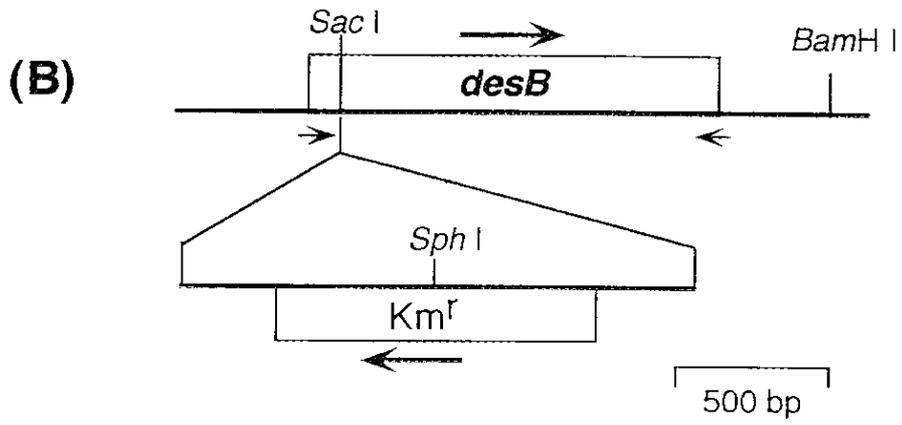
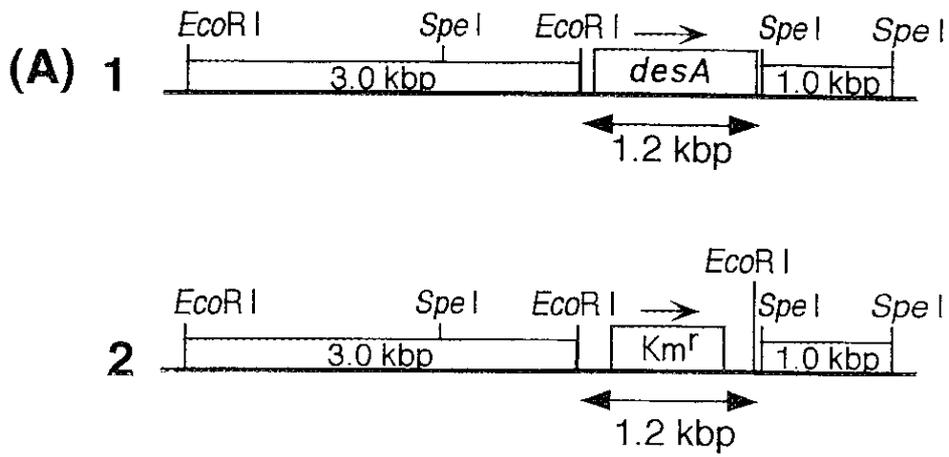


Figure 3-1. Constructs of DNA fragments for the mutation of *Synechocystis* PCC6803 and *Synechococcus* PCC7942. (A) Replacement of the *desA* gene by the Km^r cartridge. The 3.0-kbp fragment upstream and the 1.0 kbp fragment downstream of *desA* gene were combined with the Km^r of 1.2 kbp as illustrated in A2. This construct, as an insert of 5.2 kbp between the *Hinc* II and *Spe* I sites of the pBluescript II KS(+), was used to transform the wild-type strain of *Synechocystis* PCC6803 to yield the *desA*- Δ mutant by homologous recombination. (B) The insertional disruption of the *desB* gene with the Km^r cartridge. The Km^r cartridge of 1.2 kbp was inserted into the *Sac* I site in the open reading frame of the *desB* gene cloned in pBluescript II KS(+). The resultant construct was used to transform the wild-type strain of *Synechocystis* PCC6803 to generate the WT/*desB*:: Km^r mutant by homologous recombination. Small arrows indicate primers for the polymerase chain reaction. (C) The insertion of the *desA* gene and the Km^r into the *cmpA* gene of *Synechococcus* PCC7942. The *desA* gene and the Km^r cartridge were inserted into the *EcoR* I site of the *cmpA* gene (Omata et al. 1990) that had been cloned in pBluescript II KS(+). The resultant construct was used to transform the wild-type strain of *Synechococcus* PCC7942 to generate the 7942/*desA* transformant. Small arrows indicate primers for the polymerase chain reaction.

Bluescript/1.5 kbp which contained the *desA* gene (position 416 to position 661 in the nucleotide sequence deposited in the EMBL data bank with the accession number, X53508), and it was labeled with [α - 32 P]dCTP using the random primer labeling kit (Takara). After hybridization, the membranes were washed at 50°C with 0.015 M sodium citrate buffer (pH 7.0) that contained 0.15 M NaCl and 0.5% SDS, and then they were exposed to X-ray films (WIF50; Konica, Tokyo, Japan).

Nucleotide and deduced amino-acid sequences---The *EcoR* I DNA fragment of 6.6 kbp from one of the positive clones was subcloned into the *EcoR* I site of pBluescript II KS(+). The resultant plasmid was termed pBluescript/6.6-kbp. The nucleotide sequence of the 1.7-kbp region, which contained the region that hybridized with the *desA* probe of 0.25 kbp, was determined by the dideoxy chain-termination method (Sanger *et al.* 1977) using 7-deaza dGTP DNA sequencing kit (Takara), BcaBEST dideoxy sequencing kit (Takara) and 7-deaza dGTP sequenase kit (U.S. Biochemical Corporation, Cleveland, OH, USA). The entire sequences of both strands in this region were determined. The alignment of amino acid sequences was performed using the molecular evolutionary analysis system for DNA and amino-acid sequences, ODN, at National Institute of Genetics (Japan).

Disruption of the desB gene in Synechocystis PCC6803---A disrupted *desB* gene, designated *desB::Km^r*, was constructed by inserting the Km^r cartridge (described above) into the *Sac* I site of the coding region of the *desB* gene, as illustrated in

Fig. 3-1B, as follows. The plasmid containing the *desB* gene in an insert of 3.5 kbp was prepared by digestion of pBluescript/6.6-kbp with *Bam*H I and subsequent self-ligation. The resultant plasmid was partially digested with *Sac* I, blunt-ended by T4 polymerase and ligated with the Km^r cartridge. The wild-type strain of *Synechocystis* PCC6803 was transformed with the plasmid DNA that included the disrupted *desB* gene as described by Golden et al. (1987). The resultant transformant was designated WT/*desB*:: Km^r . The genomic DNA of WT/*desB*:: Km^r was analyzed by the polymerase chain reaction using the primers derived from the *desB* gene (the forward primer, 5'-TTACCATTACCTCCAA-3'; the reverse primer 5'-TTCAATTAGGATCAATTAAG-3', indicated by arrows in Fig. 3-1B). Products of the polymerase chain reaction were analyzed by agarose gel electrophoresis.

Integration of the desA gene into the chromosome of Synechococcus PCC7942---The author generated a transformant, designated 7942/*desA*, in which the *desA* gene was integrated in the chromosome of *Synechococcus* PCC7942, by integrating the *desA* gene and the Km^r cartridge into the *Eco*R I site of the coding region of the *cmpA* gene, as illustrated in Fig. 3-1C. The *cmpA* gene in the genome of *Synechococcus* PCC7942 (Omata et al. 1990) was amplified by the polymerase chain reaction using the primers for the *cmpA* (the forward primer, 5'-ATGAACGAATTTCAACCAGT-3'; the reverse primer, 5'-TGCCCGTTTAATCTTCAAGC-3'; indicated by small arrows in Fig. 3-1C). The resultant product was subcloned into the *Sma* I site

of modified pBluescript II KS(+), in which the *EcoR* I site had been deleted from the multicloning site by digestion with *Xho* I and *Pst* I, and the product was blunt-ended by T4 DNA polymerase with subsequent self-ligation. The resultant plasmid was designated pBluescript/*cmpA*. A 1.2-kbp fragment containing the *desA* gene was prepared by digesting Bluescript/1.5 kbp (Wada *et al.* 1990) with *EcoR* I and *Sma* I and blunt-ending it with T4 DNA polymerase. This fragment was inserted into the *Sma* I site of pBluescript/S1, which contained the 1.0-kbp fragment downstream of the *desA* gene in the genome of *Synechocystis* PCC6803. The resultant plasmid was designated pBluescript/*desA*/S1. pBluescript/E3/ Km^r described above, was digested with *Sma* I and *Sal* I to produce the 4.2-kbp fragment that contained the 3.0-kbp fragment and the 1.2-kbp Km^r cartridge. This fragment was blunt-ended with T4 DNA polymerase and was inserted into the *Xho* I site of pBluescript/*desA*/S1, which had also been blunt-ended by T4 DNA polymerase. The resultant plasmid was designated pBluescript/E3/ Km^r /*desA*/S1. pBluescript/E3/ Km^r /*desA*/S1 was digested with *Sma* I and *Xho* I to prepare the 2.4-kbp fragment that contained the *desA* gene and the Km^r cartridge. After blunt-ending by T4 DNA polymerase, the 2.4-kbp fragment was inserted into the *EcoR* I site of pBluescript/*cmpA* which had also been blunt-ended by T4 DNA polymerase. The resultant plasmid, containing the construct illustrated in Fig. 3-1C, was designated pBluescript/*cmpA*::*desA*.

The wild-type strain of *Synechococcus* PCC7942 was transformed with the pBluescript/*cmpA::desA* by the method of Williams and Szalay (1983). The genomic DNA from the resultant transformant, 7942/*desA*, was analyzed by the polymerase chain reaction using the *cmpA* primers described above. Products of the polymerase chain reaction were analyzed by agarose gel electrophoresis.

Introduction of the desB gene into Synechococcus PCC7942---
The 6.6-kbp *EcoR* I fragment of pBluescript/6.6-kbp, containing the *desB* gene, was subcloned into the *EcoR* I site of pUC303, a shuttle vector between *Escherichia coli* and *Synechococcus* PCC7942 (Kuhlemeier et al. 1983). The resultant plasmid, designated pUC303/6.6-kbp, was used to transform the wild type and the transformant 7942/*desA*. The transformation was accomplished essentially as described by Williams and Szalay (1983). Streptomycin-resistant transformants were selected on BG-11 plates that contained 10 $\mu\text{g ml}^{-1}$ streptomycin. The transformants of wild-type and 7942/*desA* cells were designated transformant 7942/*desB* and double-transformant 7942/*desA/desB*, respectively.

*Analysis of fatty acids---*Cells collected by centrifugation were lyophilized and subjected to methanolysis with 5% HCl (w/w) in methanol at 85°C for 2.5 h. The resultant methyl esters were extracted by *n*-hexane and analyzed by gas-liquid chromatography as described previously (Wada and Murata 1989). Methyl esters of fatty acids were identified by a

gas-liquid chromatograph and a gas chromatograph-mass spectrometer (Wada and Murata 1989).

Results

Evaluation of the desA-Δ mutant---The *desA-Δ* mutant was produced as described in Materials and Methods. Since *Synechocystis* PCC6803 contains six to eight copies of its chromosomal DNA per cell (Williams 1988), it was important to confirm that the *desA* gene had been replaced by the Km^r cartridge in the *desA-Δ* mutant in all the copies of the chromosomal DNA. The genomic DNA from wild-type and *desA-Δ* cells was analyzed by DNA/DNA blot hybridization with the probes derived from the *desA* gene and the Km^r cartridge. The DNA from the wild-type cells hybridized with the *desA* probe of 1.2 kbp giving a band of 1.1 kbp. No band was detected with the Km^r probe (Fig. 3-2A). The DNA from the *desA-Δ* mutant hybridized with the Km^r probe giving a band of 1.3 kbp, but it did not hybridized with the *desA* probe (Fig. 3-2A). These results indicate that the *desA* gene had been replaced by the Km^r cartridge in all copies of the chromosomal DNA in the *desA-Δ* mutant.

In the *desA-Δ* mutant, levels of 18:1(9) and 18:2(6,9) were significantly increased whereas 18:3(6,9,12) and 18:2(9,12) had disappeared (data not shown). The fatty acid composition of the *desA-Δ* mutant was similar to that of the Fad12 and WT/*desA::Km^r* mutants (Wada and Murata 1989, Wada *et al.* 1990), which are defective in fatty acid desaturation at the Δ12 position. This result indicates that the *desA-Δ*

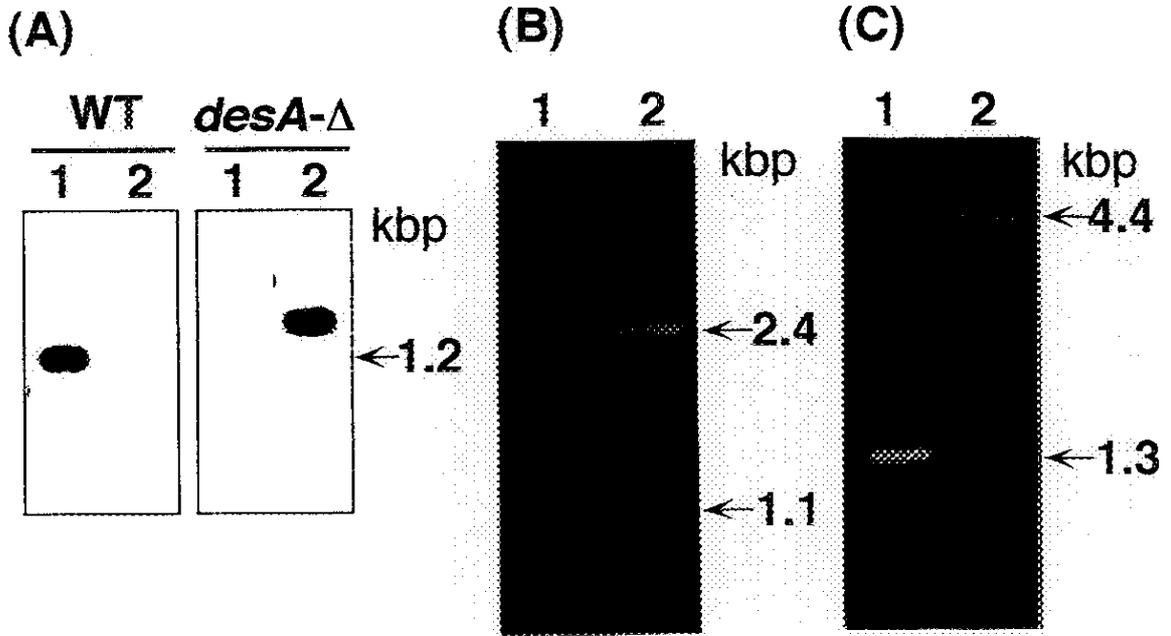


Figure 3-2. Evaluation of the successful replacement of genes in the chromosomes of *desA*- Δ , WT/*desB*::Km^r and 7942/*desA* cells. (A) Analysis by DNA/DNA hybridization to demonstrate the complete replacement of the *desA* gene by the Km^r cartridge in *desA*- Δ cells. The genomic DNA from wild-type and *desA*- Δ cells of *Synechocystis* PCC6803 was digested with *Eco*R I and *Spe* I and blotted on nylon membranes. The 1.2-kbp *desA* probe (lane 1) and the 1.2-kbp Km^r probe (lane 2) were used for hybridization. (B) Analysis by polymerase chain reaction to demonstrate the complete replacement of the native *desB* gene by the disrupted *desB* gene in WT/*desB*::Km^r cells. Genomic DNA from wild-type (lane 1) and WT/*desB*::Km^r cells (lane 2) was used as template for the polymerase chain reaction with primers indicated by small arrows in Fig. 3-1B. (C) Analysis by the polymerase chain reaction to demonstrate the integration of the *desA* gene and the Km^r cartridge into the *cmpA* gene in 7942/*desA* cells. Genomic DNA isolated from wild-type (lane 1) and 7942/*desA* cells (lane 2) of *Synechococcus* PCC7942 was used as the templates for the polymerase chain reaction with primers indicated by small arrows in Fig. 3-1C.

mutant had lost the ability to desaturate fatty acids at the $\Delta 12$ position.

Cloning of the desB gene from Synechocystis PCC6803---The genomic DNA library of the *desA*- Δ mutant, constructed in the phage vector λ DASH II, was screened with the *desA* probe of 0.25 kbp as described in Materials and Methods. About 260 putative positive clones were obtained from 3.5×10^3 recombinants, and 26 clones were arbitrarily selected among these positive clones. The restriction map and the size of the DNA fragment that hybridized with the screening probe allowed us to divide them into seven groups. The author found that one of these seven groups corresponded a desaturase. A clone of this group gave a fragment of 6.6 kbp upon digestion with *EcoR* I. The *EcoR* I fragment of 6.6 kbp was subcloned into the *EcoR* I site of pBluescript II KS(+). The nucleotide sequence of 1.7 kbp in the hybridizable region of the fragment was determined. The nucleotide sequence of 1725 bp is represented in Appendix D. The open reading frame of 1077 nucleotides was found, which corresponds to a polypeptide of 359 amino-acid residues with an approximate molecular mass of 41.9 kDa. As confirmed below, this open reading frame corresponds to the gene for the $\omega 3$ desaturase. This gene was designated *desB*.

Disruption of the desB gene in Synechocystis PCC6803---In order to confirm that the *desB* gene in WT/*desB*:: Km^r had been disrupted by insertion of the Km^r cartridge in all the copies of chromosomal DNA, genomic DNA from wild-type and

WT/*desB*::Km^r cells was analyzed by the polymerase chain reaction. A DNA fragment of 1.1 kbp, which originated from the native *desB* gene, was amplified by the polymerase chain reaction with the DNA from the wild-type cells (Fig. 3-2B, lane 1). A DNA fragment of 2.4 kbp, which was expected to include the *desB* gene that had been disrupted by insertion of the Km^r cartridge, was amplified by the polymerase chain reaction with the DNA from the WT/*desB*::Km^r cells (Fig. 3-2B, lane 2). These results indicate that the *desB* gene in WT/*desB*::Km^r was disrupted by the insertion of the Km^r cartridge.

Fatty acid compositions of the total lipids from wild-type and WT/*desB*::Km^r strains are compared in Table 3-1. Wild-type cells grown at 34°C contained 18:1(9), 18:2(9,12) and 18:3(6,9,12) as major fatty acids. The fatty acid composition of WT/*desB*::Km^r cells grown at 34°C was the same as that of the wild-type cells. When the wild-type cells were grown at 22°C, 18:3(9,12,15) and 18:4(6,9,12,15) appeared in addition to the fatty acids present at 34°C. However, these two fatty acids did not emerge in WT/*desB*::Km^r cells grown at 22°C. This result implies that the WT/*desB*::Km^r strain had lost the ability to introduce a double bond into the ω3 position of fatty acids, moreover, that the *desB* gene is responsible for the desaturation of fatty acids at the ω3 position.

Transformation of Synechococcus PCC7942 with the desA and desB genes---First, wild type of *Synechococcus* PCC7942 were

Table 3-1. Changes in composition of fatty acids of total lipids by disruption of the *desB* gene in *Synechocystis* PCC6803.

Strain	Fatty acid								
	16:0	16:1	18:0	18:1	18:1	18:2	18:3	18:3	18:4
		(9)		(11)	(9)	(9,12)	(6,9,12)	(9,12,15)	(6,9,12,15)
	(mol%)								
Grown at 34°C									
Wild type	60±1	4±1	1	1	11±1	13±1	10±1	0	0
WT/ <i>desB</i> ::Km ^r	62±2	5±1	1	1	9±1	12±1	10±1	0	0
Grown at 22°C									
Wild type	55±1	3±1	1	1	4±1	10±1	16±2	6±1	4±1
WT/ <i>desB</i> ::Km ^r	54±2	5±1	1	1	4±1	16±2	19±2	0	0

Values were obtained from three independent cultures.

transformed with the *desA* gene and the Km^r cartridge, which had been integrated into the *cmpA* gene as in Fig. 3-1C. In order to confirm the integration of this construct into the *cmpA* gene of the genome, the genomic DNA from wild-type and 7942/*desA* cells was analyzed by the polymerase chain reaction. A DNA fragment of 1.3 kbp, which originated from the native *cmpA* gene, was amplified from the DNA of the wild-type cells (Fig. 3-2C, lane 1). By contrast, a DNA fragment of 4.4 kbp, which was expected from the *cmpA* gene modified by integration of the *desA* gene and the Km^r cartridge, was amplified from the DNA of the 7942/*desA* cells (Fig. 3-2C, lane 2). A DNA fragment of 1.3 kbp, corresponding to the native *cmpA* gene, was not detected. These findings suggest that both the *desA* gene and the Km^r cartridge had been successfully inserted into the *cmpA* gene of the 7942/*desA* strain.

Wild-type and 7942/*desA* cells were transformed with the pUC303/6.6-kbp that contained the *desB* gene from *Synechocystis* PCC6803, as described in Materials and Methods. The transformants of wild-type and 7942/*desA*, designated 7942/*desB* and 7942/*desA/desB*, respectively, were isolated and examined.

Fatty acid compositions of total lipids from wild-type, 7942/*desA*, 7942/*desB* and 7942/*desA/desB* cells are shown in Table 3-2. The wild-type cells grown at 34°C contained saturated and monounsaturated fatty acids, such as 16:0, 16:1(9), 18:1(9) and 18:1(11). In 7942/*desA* cells, 16:2(9,12) and 18:2(9,12) emerged with decreases in the

Table 3-2. Changes in composition of fatty acids of total lipids by transformation of *Synechococcus* PCC7942 with the *desA* and *desB* genes.

Strain	Fatty acid									
	16:0	16:1 (9)	16:2 (9,12)	16:3 (?)	18:0	18:1 (11)	18:1 (9)	18:2 (9,12)	18:2 (?)	18:3 (9,12,15)
(mol%)										
Grown at 34°C										
Wild type (7942)	54 [±] 3	34 [±] 2	0	0	4 [±] 1	3 [±] 1	5 [±] 1	0	0	0
Transformants										
7942/ <i>desA</i>	54 [±] 2	25 [±] 1	9 [±] 1	0	3 [±] 1	3 [±] 1	t	4 [±] 1	2 [±] 1	0
7942/ <i>desB</i>	53 [±] 2	36 [±] 2	0	0	2 [±] 1	6 [±] 1	3 [±] 1	0	0	0
7942/ <i>desA/desB</i>	56 [±] 2	24 [±] 1	8 [±] 1	1	4 [±] 1	3 [±] 1	t	3 [±] 1	2 [±] 1	1
Grown at 34°C, then incubated at 22°C for 18 h										
Wild type (7942)	46 [±] 1	40 [±] 2	0	0	3 [±] 1	6 [±] 1	4 [±] 1	0	0	0
Transformants										
7942/ <i>desA</i>	45 [±] 2	18 [±] 2	14 [±] 2	0	4 [±] 1	5 [±] 1	t	7 [±] 1	8 [±] 2	0
7942/ <i>desB</i>	50 [±] 2	35 [±] 2	0	0	4 [±] 1	6 [±] 1	6 [±] 1	0	0	0
7942/ <i>desA/desB</i>	47 [±] 1	18 [±] 2	11 [±] 1	4 [±] 1	4 [±] 1	5 [±] 1	t	3 [±] 1	4 [±] 1	5 [±] 1

16:3(?) and 18:2(?) are hexadecatrienoic and octadecadienoic acids, respectively, but the sites of their double bonds were not determined.

t, trace amount (less than 0.5%).

Values were obtained from three independent experiments.

levels of 16:1(9) and 18:1(9), respectively. By contrast, the fatty acid composition of 7942/*desB* cells was the same as that of wild-type cells. 7942/*desA/desB* cells contained 18:3(9,12,15) in addition to 16:2(9,12) and 18:3(9,12). Incubation of these cells at 22°C for 18 h enhanced the desaturation of 16:1(9) and 18:1(9) in 7942/*desA* cells, and of 18:2(9,12) in 7942/*desA/desB* cells. These results indicate that the 7942/*desA/desB* strain acquired the ability to introduce a double bond at the ω 3 position of fatty acids upon transformation with both *desA* and the *desB* gene. The cells transformed with the *desB* gene alone, 7942/*desB*, was unable to desaturate fatty acids at the ω 3 position.

Discussion

In the present study, the author isolated the gene for the ω 3 desaturase from *Synechocystis* PCC6803. Using a technique for the insertional disruption of genes, the author produced the mutant with disrupted *desB* gene that was unable to desaturate fatty acids at the ω 3 position. By introduction of genes for desaturase into *Synechococcus* PCC7942, the author produced transformant capable of desaturating fatty acids at the ω 3 position. This result indicates that the electron-donating system in *Synechococcus* PCC7942 can operate to support the function of the ω 3 desaturase of *Synechocystis* PCC6803 regardless the reaction specificity of the enzyme.

Figure 3-3 shows the alignment of the amino acid sequence deduced from *desB* with those deduced from *ARG1* cDNA, which was obtained as the cDNA analog of an auxin-regulated

desB VRLEISSPQTKLPYPKTEELPFTLQELRNAIPADCFEPSVVRSLGYFFLDV 51
ARG1 MIQAQTLQHFNGAREGDQSYFDPGAPP..KIADI.A...KH.W.K.TL...S.VLR.. 59
fad3 MVVAMDQRSNVNGD.GARKEEGFDPSAQP..KIGDI.A...KH.WVK.PL..MS.VTR.I 60
desA MTATIPPLT.TVTPSN.DRPIADLK..DIIKTL.KE...KKASKAWASVLITL 53

* *

desB GLIAGFYALAAAYLDSWFFYPFIWFLIQGTLFWSL FVVGHDCGHSFS KSKTLNNWIGHLSH 111
ARG1 LVVTALA.S.ISFN...W.LY.PA...M..A...L.....N.SK..SFV..IL. 119
fad3 FAV.ALAMA.V.F...LW.LY.VA.....AI..L.....DIPL..SVV..IL. 120
desA .A..VG.LGII..-P.YCL..T.IWT..ALTGA.....R..A.KRWV.DLV..IAF 112
 * * * * * ** * * * * * ** * *

desB TP ILVPHYGWRISHRTHHANTG NIDTDESWYPVSEQKYNQMAWYEKLLR--FYLPLIAYP 169
ARG1 SLN.....Q.H.HVEK....V.LT.KV.KNLDDMTRM..YS.PF.IF... 179
fad3 SFQ.H.HVEN....V.LP.KL.KNLPHSTRM..YTVP..ML... 180
desA A.LIY.F.S..LL.DH..LH.NK.EV.NA.D.W.VEAFQASPAIVR.-----FYRA.RG. 167
 * ** * ** * * * *

desB IYLFRRSPNRQGSHFMPGSPLFRPGEKAAVLTSTFALAAFVGLGFLTWQFGWLFLLKFY 229
ARG1 F..WN...GKE...N.Y.N..S...RKG.V...LCWGIVLSV.LY.SLTI.PI.M..L. 239
fad3 ..WY...GKE...N.Y.S..A.S.RKLI...TCWSIMLAT.VY.SFLVDPVTV..V. 240
desA FWWTGSIFHWSLM..KLSNFAQ.DRN.VKLSIAVVF.F.AIA.PA-.IITT.VWGFV..W 226
 ** *

desB VAPYLVFVVWLDLVTFLHHT--EDNIPWYRGDDWYFLKALSTIDR-DYG-FINPI HHHDI 285
ARG1 GV...I..M...F..Y...HGYTHKL.....QE.SY.R.G.T.V...-...-W..NV.... 297
fad3 GV..II..M...A..Y...HGHDEKL.....KE.SY.R.G.T.....-...-IF.N.... 298
desA LM.W..YHF.MSTF.IV...--IPE.RFRPAA..SAAEAQ.NGTVHC..PRWVEVL C... 284
 * * * * * * * * * *

desB GTHVAHHIF SNMPHYKLRRATEAIKPILGEYYRYSDEP-----IWQAFFKSYWACHFVP 339
ARG1I..L.PQI...H.VE..KSA.SV..K...EPQKSGPLPFHLLKYLLQ.ISQD...S 357
fad3I..L.PQI...H.VD..R.A.HV..R...EPKTSGAIPHLVESLVA.IKKD.Y.S 358
desA NV.IP..LSVAI.S.N..L.HGSL.ENW.PFL-.ERTF-----N..LMQQISGQ..LYD 337
 * ** * * * * *

desB NQSGVYYYQSPSNGGYQKQP 359
ARG1 DT.DI....TDPKLHQDSWTKSK 380
fad3 DT.DI.F.ETDPDLVYASDKSKIN 383
desA PEHGYRTFGSLKKV 351

Figure 3-3. Alignment of amino-acid sequences deduced from the *desB* gene, *ARG1* cDNA (Yamamoto et al. 1992), *fad3* cDNA (Arondel et al. 1992) and the *desA* gene (Wada et al. 1990). The conserved amino acids in all sequences are indicated by asterisks. The domains conserved in the ω 3 desaturases are boxed. The domains conserved in the Δ 12 desaturases of cyanobacteria (described in Chapter 2) are indicated by underlining. The nine amino-acid sequence, conserved in the products of the *desA* and the *desB* genes of *Synechocystis* PCC6803, is indicated by bar. The histidine residues conserved in the products of the *desA* and the *desB* genes of *Synechocystis* PCC6803 are indicated by arrowheads.

gene of mung bean (Yamamoto *et al.* 1992, Yamamoto 1993), *fad3* cDNA that encodes an ω 3 desaturase of *Brassica napus* (Arondel *et al.* 1992), and *desA* that encodes the Δ 12 desaturase of *Synechocystis* PCC6803 (Wada *et al.* 1990). The extents of similarity in terms of amino acid sequence between the product of the *desB* gene and those of the *ARG1* cDNA, the *fad3* cDNA, and the *desA* gene, are 44%, 42% and 27%, respectively. Three domains (Fig. 3-3, indicated by boxes) are conserved among these ω 3 desaturases. The first conserved domain of the ω 3 desaturases is similar to the corresponding domain of the Δ 12 desaturase of *Synechocystis* PCC6803 (Wada *et al.* 1990). The first conserved domain is also conserved in the Δ 12 desaturases of four strains of cyanobacteria (Fig. 3-3, indicated by underlining). A short region of consecutive amino-acid identity in the first conserved domain (Fig. 3-3, indicated by bar) was found in the ω 3 desaturase and the Δ 12 desaturase of *Synechocystis* PCC6803. However, the amino acid sequences of the second and third domains of the ω 3 desaturases differ from those of the corresponding domains of the Δ 12 desaturase of *Synechocystis* PCC6803. It is likely that the first domain plays an essential role in the desaturation of fatty acids bound to membrane lipids, while the second and the third domains are essential for the introduction of a double bond at the ω 3 position of fatty acids.

Striking similarities were also found between the hydrophobic characteristics of the products of the *desB* and *desA* genes (Fig. 3-4). The predicted product of the *desB*

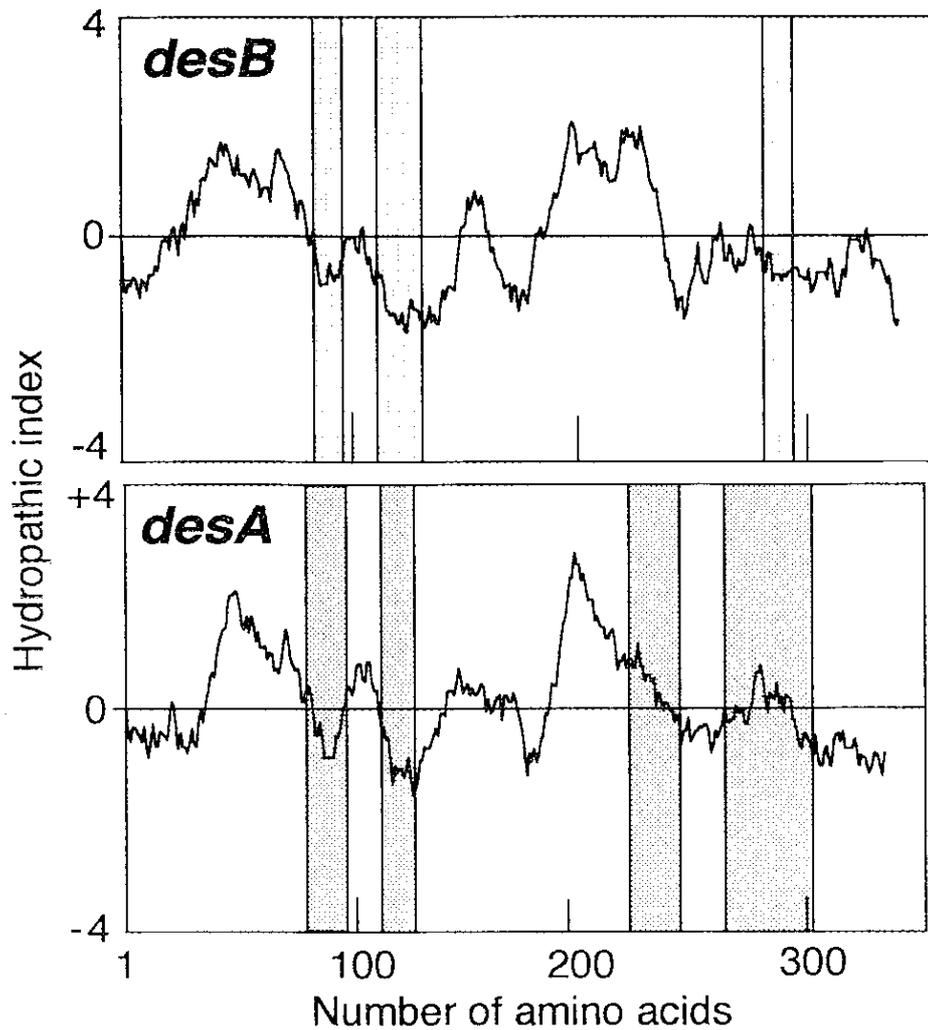


Figure 3-4. Hydropathy profile of the product of the *desB* gene, compared with that of the *desA* gene. The domains conserved in the ω 3 desaturases and those in the Δ 12 desaturases of cyanobacteria (described in Chapter 2) are indicated by shadowing. The hydropathy index was calculated by use of the algorithm of Kyte and Doolittle (1982) for a window size of 19 amino-acid residues.

gene contains two hydrophobic region, and, therefore, it is likely to be a membrane-bound protein. The three conserved domains (Fig. 3-4, indicated by shadowing) are all localized in the hydrophilic region. The first conserved domain, that includes the nine amino-acid sequence conserved between $\Delta 12$ and $\omega 3$ desaturases of *Synechocystis* PCC6803 (Fig. 3-3; indicated by bar), is located at the border between the first hydrophobic region and the next hydrophilic region. The hydropathy profiles of the $\omega 3$ desaturases from higher plants are similar to that of the product of the *desB* gene (data not shown).

Recently, a gene for the $\Delta 6$ desaturase was cloned from *Synechocystis* PCC6803 (Reddy et al. 1993). The similarity between the deduced amino-acid sequence of the $\Delta 6$ desaturase and that of the product of the *desB* gene is very small, namely 16%. However, the hydropathy profile of the $\Delta 6$ desaturase is similar to that of the product of the *desB* gene (data not shown).

No similarity was found between the amino acid sequence deduced from the *desB* gene and those of the 18:0-CoA desaturases from rat (Thiede et al. 1986), mouse (Ntambi et al. 1988) and *S. cerevisiae* (Stukey et al. 1990). In spite of the lack of sequence similarity, the hydropathy profiles of the 18:0-CoA desaturases are similar to that of the product of the *desB* gene (data not shown). The $\Delta 9$ 18:0-ACP desaturases from castor bean (Shanklin and Somerville 1991, Knutzon et al. 1991), safflower (Thompson et al. 1991), cucumber (Shanklin et al. 1991), spinach (Nishida et al.

1992) and rape seed (Knutzon *et al.* 1992), and the $\Delta 6$ 18:0-ACP desaturase from coriander (Cahoon *et al.* 1992) are different from the product of the *desB* gene in terms of both amino acid sequence and hydropathy profile (data not shown).

Histidine residues are well conserved in the $\omega 3$ and $\Delta 12$ desaturases of *Synechocystis* PCC6803 (Fig. 3-3, indicated by arrowheads). Ten of 14 histidine residues are located in the three domains that are conserved among the $\omega 3$ desaturases. It is notable that histidine is the amino acid residue conserved between the 18:0-CoA desaturases from rat (Thiede *et al.* 1986) and *S. cerevisiae* (Stukey *et al.* 1990). Although the role of the histidine residues in the desaturation of fatty acids has not been clarified, the author can assume that these residues contribute to the catalytic function of the desaturases.

The level of the *desB* transcript was about ten times higher in cells incubated at low temperatures, such as 22°C, 24°C, 26°C, than that in cells grown at 34°C (data not shown). This result is compatible with the results of the analysis of fatty acid composition, namely, 18:3(9,12,15) and 18:4(6,9,12,15) accumulated in cells grown at low temperatures (Wada and Murata 1990). The low-temperature-induced accumulation of the *desB* transcript is also compatible with recent findings that the transcription of the *desA* gene is accelerated by a decrease in temperature (Los *et al.* 1993), as well as by hydrogenation of lipids of the plasma membrane (Vigh *et al.* 1993).

Recently, a sequence motif conserved in low-temperature-inducible promoters of *E. coli*, CCAAT, was identified by comparing the 5' upstream nucleotide sequences of low-temperature-inducible genes (Qoronfleh et al. 1992). Since the motif, CCAAT, was not found in the 5' non-coding region of the *desB* gene, the mechanisms for the induction of the *desB* gene at low temperature may be different from that of induction of the low-temperature-inducible genes of *E. coli*.

In 7942/*desA* cells, namely of *Synechococcus* PCC7942 cells transformed with the *desA* gene, the *desA* gene and the Km^r cartridge were inserted into the *cmpA* gene. It had been shown (Omata et al. 1990) that there are no significant differences between wild-type and mutant cells with a disrupted *cmpA* gene in terms of growth rates under either low or high CO₂ conditions. Therefore, it is unlikely that the disruption of the *cmpA* gene itself does affect the desaturation of fatty acids.

The introduction of the double bond at the ω 3 position occurred in 7942/*desA*/*desB* cells, namely *Synechococcus* PCC7942 cells transformed with both the *desA* and the *desB* genes, but not in 7942/*desB* cells, the transformant that harbored the *desB* gene alone. This result suggests that a double bond at the ω 3 position cannot be introduced when the fatty acids do not contain a double bond at the Δ 12 position. This observation is compatible with the observation that the Fad12 mutant of *Synechocystis* PCC6803, which is defective in desaturation at the Δ 12 position, cannot synthesize fatty acids which have a double bond at the ω 3 position (Wada and

Murata 1989). It is unclear how the ω 3 desaturase identifies the exact position for its desaturation of fatty acids. The information of the structure of cyanobacterial desaturases should provide clues towards an answer to this question.

Chapter 4

Molecular and functional characterization of the $\Delta 9$
desaturase of cyanobacteria.

Abstract

In cyanobacteria, 18:1(9) is synthesized by introduction of a double bond at the $\Delta 9$ position of 18:0 by a membrane-bound acyl-lipid desaturase. The author isolated the genes, designated *desC*, for the $\Delta 9$ desaturase from *Anabaena variabilis* and *Synechocystis* PCC6803. Upon expression of the *desC* gene of *Synechocystis* PCC6803 in *Escherichia coli*, the *E. coli* cells accumulated 18:1(9), which is absent in the wild-type cells of *E. coli*. The amino acid sequences of the $\Delta 9$ desaturase deduced from the nucleotide sequences of the *desC* genes of *A. variabilis* and *Synechocystis* PCC6803 are similar, to some extent, to the sequences of 18:0-CoA desaturases of rat, mouse and *Saccharomyces cerevisiae*.

Introduction

Unsaturated fatty acids are constituent of polar glycerolipids of biological membranes and the unsaturation of membrane lipids is important to the fluidity of membranes at a suitable level (Chapman 1975). It has been demonstrated that the unsaturation of membrane lipids is essential for the low-temperature tolerance of cyanobacteria (Wada *et al.* 1990, Wada *et al.* 1994, Gombos *et al.* 1992).

There are two principal pathways for the synthesis of unsaturated fatty acids, i.e., the aerobic and the anaerobic pathways (Stumpf 1980). In the aerobic pathway, double bonds are introduced after fatty acids synthesized. The fatty-acid desaturases are enzymes which catalyze this oxidative introduction of double bonds into fatty acids (Jaworski 1987). In the anaerobic pathway, a double bond is introduced during the synthesis of fatty acids (Bloch 1971).

In the cyanobacterial cells, desaturations of fatty acids take place via the aerobic pathway after the fatty acids are bound to glycerolipids (Sato and Murata 1982a,b, Sato *et al.* 1986). Wada *et al.* (1993) demonstrated that thylakoid membranes isolated from the cyanobacterium, *Synechocystis* PCC6803, were capable of desaturating 18:0 to 18:1(9) in monogalactosyl diacylglycerol.

In higher plants, the desaturation of 18:0 to 18:1(9) occurs in the ACP bound form (McKeon and Stumpf 1982, Stumpf 1981), whereas all the other desaturation reactions take place after fatty acids are become to glycerolipids (Harwood

1988, Jaworski 1987). The enzyme in higher plants, which catalyzes the desaturation of 18:0-ACP, is a water-soluble protein localized in the stroma of the chloroplast. In animal and fungal cells, the desaturation of fatty acids occurs in the CoA bound form (Holloway 1983). The enzyme in mammals, which catalyzes the desaturation of 18:0-CoA, is a membrane-bound protein localized in the endoplasmic reticulum (Strittmater et al. 1974).

In the cyanobacterium, *Synechocystis* PCC6803, unsaturated fatty acids are synthesized by sequential introduction of double bonds into 18:0 esterified to the *sn*-1 position of glycerolipids by four distinct desaturases, which catalyze the desaturation of fatty acids at their own specific positions (Murata et al. 1992b, Wada and Murata 1989, 1990). The study of substrate specificity of the desaturases of *Synechocystis* PCC6803 by feeding an odd-numbered aliphatic acid (Higashi and Murata 1993) demonstrated that each of the desaturases introduces a double bond at the $\Delta 6$, $\Delta 9$, $\Delta 12$ or $\omega 3$ position of fatty acids. The $\Delta 12$ desaturase gene, *desA* (Wada et al. 1990), the $\omega 3$ desaturase gene, *desB* (described in Chapter 3) and the $\Delta 6$ desaturase gene (Reddy et al. 1993) have been cloned from *Synechocystis* PCC6803. The author has isolated the $\Delta 12$ desaturase genes, *desA*, from three other cyanobacterial strains, i.e. *Synechocystis* PCC6714, *Synechococcus* PCC7002 and *Anabaena variabilis* (described in Chapter 2).

In the present study, the author found genes for the $\Delta 9$ desaturases (termed *desC*) of *A. variabilis* and *Synechocystis*

PCC6803. Their deduced amino-acid sequences are similar to 18:0-CoA desaturases from mammals and yeast. Expression of the gene of *Synechocystis* PCC6803 in *Escherichia coli* enabled the bacterium to desaturate 18:0 to 18:1(9).

Materials and Methods

Cloning of the desC gene of Anabaena variabilis---An *EcoR* I fragment of 7 kbp of the genomic DNA of *A. variabilis* (Fig. 4-1A) was cloned, and the nucleotide sequence of 2618 bp of the 7-kbp fragment of *A. variabilis* was determined, as described in Chapter 2. The author found an open reading frame (termed *desC*) at the 5' upstream region of the *desA* gene of *A. variabilis*.

Cloning of desC gene of Synechocystis PCC6803---The genomic library of the *desA-A* mutant of *Synechocystis* PCC6803 (described in Chapter 3) was screened by plaque hybridization essentially as described by Ausubel et al. (1987).

Approximately 2.5×10^3 plaques were transferred onto nylon membranes (Hybond-N+; Amersham, Buckinghamshire, UK), and hybridized at 50°C for 23 h with the 0.75-kbp probe derived from the *desC* gene of *A. variabilis* (Fig. 4-1A) in 0.09 M sodium citrate buffer (pH 7.0) containing 0.9 M NaCl, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 10% dextran sulfate, 0.1% SDS and $100 \mu\text{g ml}^{-1}$ salmon sperm DNA. The 0.75-kbp probe for the screening was prepared by digestion with *Mun* I of the plasmid containing a *Hinc* II-*Hind*

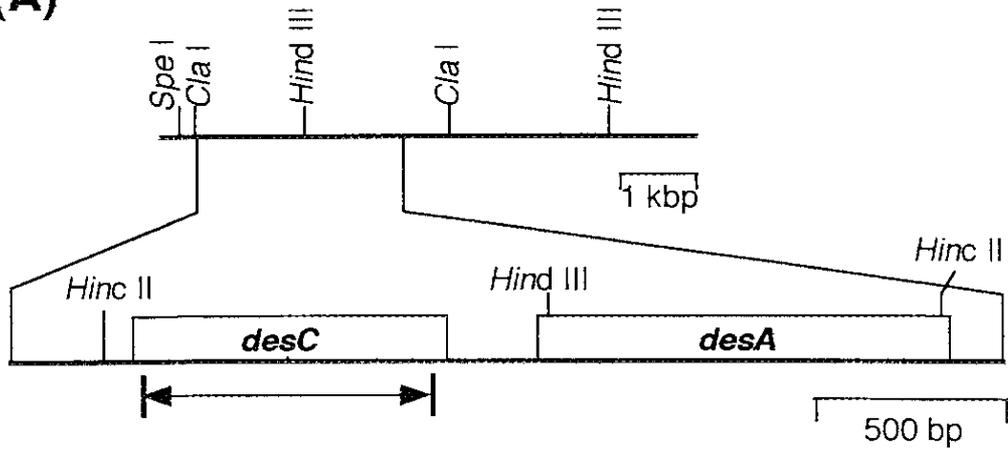
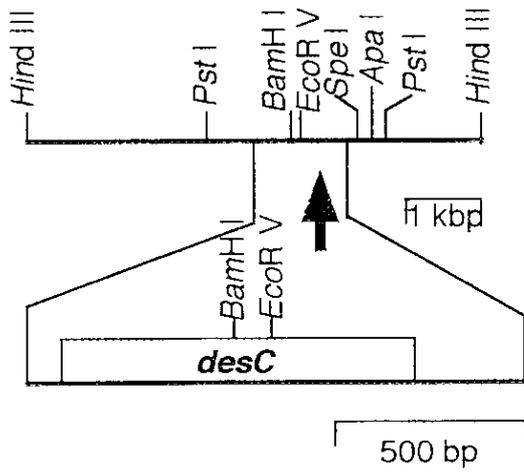
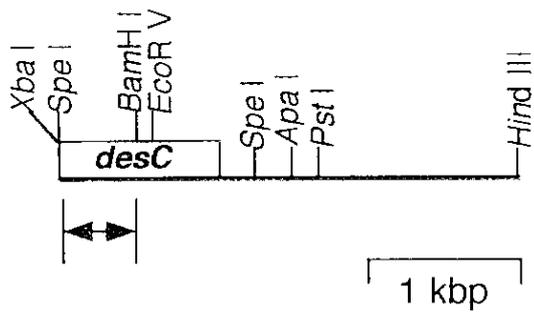
(A)**(B)****(C)**

Figure 4-1. Restriction maps for the cloned DNA fragments.

(A) The restriction map of the DNA fragment of 7 kbp containing the *desA* and *desC* genes of *A. variabilis*. The nucleotide sequence has been determined for the region of 2,618 bp (represented in Appendix C). The probe of 0.75 kbp (position 315 to position 1,070 in the nucleotide sequence in Appendix C) for the screening of the genomic library of *Synechocystis* PCC6803 is indicated by a bar. (B) The restriction map of the 6-kbp insert of the pBluescript/H6 which contained the *desC* gene of *Synechocystis* PCC6803. The region which hybridized with the 0.75-kbp probe is indicated by an arrow. The nucleotide sequence was determined for the region of 1,275 bp (represented in Appendix E). (C) The restriction map of the 3-kbp insert of the pBluescript/*desC*. The region of 0.5 kbp which was amplified by the polymerase chain reaction was indicated by a bar.

III fragment of 1.1 kbp of the *desC* gene of *A. variabilis* (Fig. 4-1A). It was labeled with [α - 32 P]dCTP using the BcaBEST labeling kit (Takara). After the hybridization, the membranes were washed at 50°C with 0.015 M sodium citrate buffer (pH 7.0) containing 0.15 M NaCl and 0.5% SDS, and then they were exposed to X-ray films (WIF50; Konica, Tokyo, Japan).

The *Hind* III DNA fragment of 6 kbp from one of the positive clones was subcloned into the *Hind* III site of pBluescript II KS(+) (Stratagene). The subclone was designated pBluescript/H6. The nucleotide sequence of 1,274 bp of the 6-kbp fragment of *Synechocystis* PCC6803, which contained the region hybridized with the 0.75-kbp probe, was determined by dideoxy chain termination method (Sanger *et al.* 1977) using BcaBEST dideoxy sequencing kit (Takara). The entire sequences of both strands in this region were determined.

Analysis of the deduced amino-acid sequence---The alignment search for the deduced amino-acid sequences of the *desC* genes of *A. variabilis* and *Synechocystis* PCC6803 was performed using the BLAST algorithm (Altschul *et al.* 1990) at the National Center for Biotechnology Information. The alignment of amino acid sequences was performed using the molecular evolutionary analysis system for DNA and amino-acid sequences, ODEN, at National Institute of Genetics.

Expression of the desC gene in Escherichia coli---The *desC* gene of *Synechocystis* PCC6803 was expressed, using a

bacteriophage T7 RNA polymerase system (Studier et al. 1990), in *E. coli*, which does not contain the $\Delta 9$ desaturase. At first, *Xba* I and *Spe* I restriction sites were introduced at the 5' end of the open reading frame of the *desC* gene of *Synechocystis* PCC6803 by amplifying the 5' half of the open reading frame of the *desC* gene by the polymerase chain reaction with the λ DNA of the positive clone as the template and the primers, 5'-GGTCTAGAATGACTAGTCCATTAAACATTGAATACCTATA and 3'-ACACCACCCCGGATCCCA. Then, the amplified fragment of 0.5 kbp was digested by *Xba* I and *Bam*H I, and ligated into the plasmid pBluescript/H6 which had been digested by *Xba* I and *Bam*H I. The resultant plasmid with the insert of the 3-kbp fragment was designated pBluescript/*desC* (Fig. 4-1C). The nucleotide sequence of the amplified region of 0.5 kbp of the pBluescript/*desC* was determined in order to confirm the nucleotide sequence was not changed by the polymerase chain reaction. The pBluescript/*desC* was digested with *Spe* I, and the resultant fragment of 1.1 kbp, containing the open reading frame of the *desC* gene, was ligated with the pET3a which had been digested by *Nhe* I. The resultant plasmid was designated pET3a/*desC*, in which the *desC* gene was constructed under control of the T7 bacteriophage promoter, $\Phi 10$, of the pET3a. However, the N-terminal sequence of the gene product was changed from M-L-N- to M-A-S-. The pET3a/*desC* and pET3a were introduced into cells of *E. coli*, BL21(DE3)pLysS.

Analysis of fatty acids in E. coli cells---*E. coli* cells transformed with pET3a/*desC* or pET3a were grown at 37°C in M9

medium supplemented with 1 mM MgSO₄, 0.2% glucose, 0.5 µg ml⁻¹ vitamin B₁, 0.1% casamino acids, 50 µg ml⁻¹ ampicillin, 20 µg ml⁻¹ chloramphenicol, 10 µM FeCl₃ and 0.1 mM sodium stearate. After the cultures reached an OD₆₀₀ of 0.6, expression of the *desC* gene was initiated upon addition of IPTG at a final concentration of 1 mM. After incubation for 1 h, the cells were collected by centrifugation, washed twice with 40 mM Tricine-KOH buffer (pH 8.0) containing 0.3 M sorbitol and 10 mM MgCl₂, lyophilized and subjected to methanolysis with 5% HCl (w/w) in methanol at 85°C for 2.5 h. The resultant methyl esters were extracted by *n*-hexane and analyzed with a gas-liquid chromatograph (GC-14A; Shimazu, Kyoto, Japan) equipped with a capillary column (25 m x 0.32 mm, Thermon 3000A). Methyl esters of fatty acids were identified by the retention time of the gas-liquid chromatography. The relative amounts of fatty acid methyl esters were calculated by comparing areas under the peaks on the chromatogram with a data processor (C-R2AX, Shimazu).

Results

The desC gene of A. variabilis---The author found an open reading frame in the 5' upstream region of the *desA* gene of *A. variabilis* (Fig. 4-1A). The open reading frame encodes a polypeptide of 272 amino-acid residues having a molecular mass of 31.4 kDa. The amino acid sequence deduced from the gene of *A. variabilis* showed significant similarity to 18:0-CoA desaturases of rat (31%), mouse (31%) and *S. cerevisiae* (29% in the internal region). Thus, the author postulated

that the gene of *A. variabilis* encodes a cyanobacterial $\Delta 9$ desaturase, and the gene was designated *desC*.

Cloning of the desC gene from Synechocystis PCC6803---The genomic DNA library of the *desA*- Δ mutant (described in Chapter 3) of *Synechocystis* PCC6803 was screened with the probe of 0.75 kbp derived from the *desC* of *A. variabilis* as described in Materials and Methods. Twenty two positive clones were obtained from 2.5×10^3 recombinants. The author selected one of the positive clones, which produced a fragment of 6 kbp with *Hind* III digestion. The *Hind* III fragment of 6 kbp was subcloned into the *Hind* III site of pBluescript II KS(+). The nucleotide sequence of 1275 bp in the hybridizable region of the fragment was determined, and an open reading frame was found (Fig. 4-1B). The nucleotide sequence of the *desC* gene of *Synechocystis* PCC6803 was represented in Appendix E. The open reading frame of 954 nucleotides encodes a polypeptide of 318 amino-acid residues with an approximate molecular mass of 37.2 kDa. The score of sequence similarity between the product of the *desC* gene of *Synechocystis* PCC6803 and that of *A. variabilis* is 62%. As confirmed below, the *desC* gene of *Synechocystis* PCC6803 encodes a $\Delta 9$ desaturase.

Expression of desC gene in E. coli---The *desC* gene was introduced into an *E. coli* strain, BL21(DE3)pLysS, under control of the T7 bacteriophage promoter, $\Phi 10$, as described in Materials and Methods. Fatty acid compositions of total lipids from the cells transformed with pET3a and pET3a/*desC*

are shown in Table 4-1. Before induction by IPTG, the cells transformed with pET3a and pET3a/*desC* contained saturated and monounsaturated fatty acids, such as 12:0, 14:0, 16:0, 16:1(9), 18:0 and 18:1(11), and the fatty acid composition were similar to each other. They contained a high level of 18:0 because of feeding stearate, although the level of 18:0 was less than 1%, when the cells transformed with pET3a and pET3a/*desC* were cultured in the M9 medium without stearate (data not shown). After induction of IPTG for 1 h, 18:1(9) emerged to a significant extent in the cells transformed with pET3a/*desC*. By contrast, the cells transformed with pET3a did not contain 18:1(9) after induction of IPTG. The results indicate that the *E. coli* cells acquired the ability to introduce a double bond at the $\Delta 9$ position of fatty acids by induction of the *desC* gene of *Synechocystis* PCC6803.

Discussion

In the present study, the genes for the $\Delta 9$ desaturase were isolated from *A. variabilis* and *Synechocystis* PCC6803. Thus, the author has obtained the structural information of the full set of the desaturases of *Synechocystis* PCC6803, each of which catalyzes the desaturation at $\Delta 9$, $\Delta 6$, $\Delta 12$ and $\omega 3$ positions of fatty acids, respectively. This enable us to compare these four types of the desaturase.

Figure 4-2 shows the alignment of the amino acid sequences deduced from the *desC* genes of *Synechocystis* PCC6803 and *A. variabilis* with those of 18:0-CoA desaturases

Table 4-1. Changes in composition of fatty acids of total lipids by introduction of the *desC* gene of *Synechocystis* PCC6803 into *E. coli*.

Transformed with	Fatty acid						
	12:0	14:0	16:0	16:1 (9)	18:0	18:1 (9)	18:1 (11)
(mol%)							
Before induction							
pET3a	2 ^{±1}	4 ^{±1}	20 ^{±1}	13 ^{±1}	44 ^{±3}	0	15 ^{±1}
pET3a/ <i>desC</i>	3 ^{±1}	5 ^{±1}	23 ^{±1}	14 ^{±1}	34 ^{±4}	0	19 ^{±2}
Induced by IPTG for 1 h							
pET3a	3 ^{±1}	6 ^{±1}	28 ^{±3}	16 ^{±2}	23 ^{±2}	0	21 ^{±3}
pET3a/ <i>desC</i>	2 ^{±1}	4 ^{±1}	27 ^{±1}	18 ^{±1}	20 ^{±2}	5 ^{±1}	22 ^{±1}

Values were obtained from three independent experiments.

from rat (Thiede *et al.* 1986), mouse (Ntambi *et al.* 1988) and *S. cerevisiae* (Stukey *et al.* 1990). The product of the *desC* gene of *Synechocystis* PCC6803 shows sequence similarity to those of the 18:0-CoA desaturases of rat (25%), mouse (24%) and *S. cerevisiae* (25% in internal region corresponding to the *desC*).

The $\Delta 9$ 18:0-ACP desaturases from castor bean (Shanklin and Somerville 1991, Knutzon *et al.* 1991), safflower (Thompson *et al.* 1991), cucumber (Shanklin *et al.* 1991), spinach (Nishida *et al.* 1992) and rape seed (Knutzon *et al.* 1992), and the $\Delta 6$ 18:0-ACP desaturase from coriander (Cahoon *et al.* 1992) are dissimilar to the product of the *desC* gene in terms of the deduced amino-acid sequence and the hydropathy profile (data not shown).

Histidine residues are well conserved in the products of the *desC* and the 18:0-CoA desaturases. Ten of 15 histidine residues of the *desC* product are conserved in the 18:0-CoA desaturases. Especially, two sets of the clusters of histidine residues, i.e. -H-X-X-H-H-, were found in the $\Delta 9$ desaturases (indicated by bar in Fig. 4-2).

The product of the *desC* gene is dissimilar to the other desaturases of *Synechocystis* PCC6803 in the amino acid sequences, i.e., the similarities are 15%, 13% and 13% to the $\Delta 6$, $\Delta 12$ and $\omega 3$ desaturase, respectively. However, hydrophobic characteristics of the four desaturases of *Synechocystis* PCC6803 resembles each other (Fig. 4-3). They have two hydrophobic regions (indicated by bar in Fig. 4-3), suggesting the translational products of these genes are

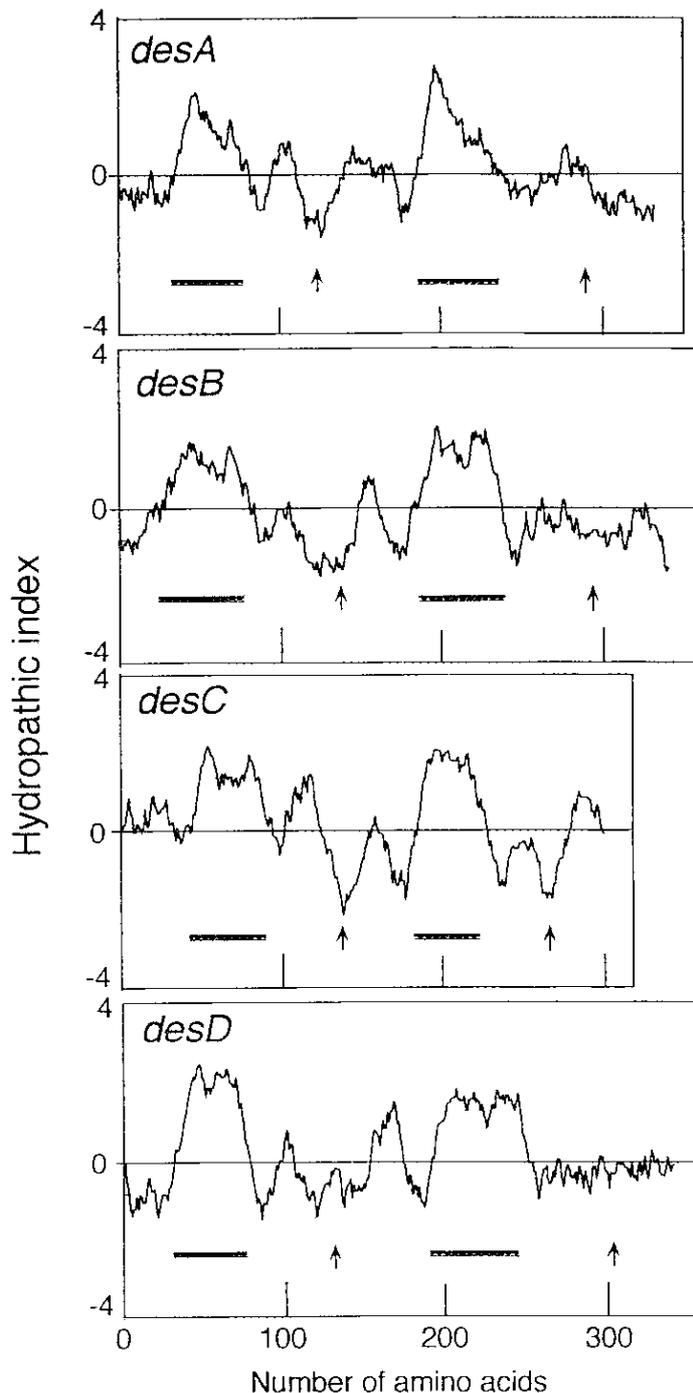


Figure 4-3. Hydropathy profiles of the four desaturases of *Synechocystis* PCC6803. *desA*, $\Delta 12$ desaturase (Wada *et al.* 1990); *desB*, $\omega 3$ desaturase (described in Chapter 3); *desC*, the $\Delta 9$ desaturase; *desD*, $\Delta 6$ desaturase (Reddy *et al.* 1993). Hydropathy index was calculated by use of the algorithm of Kyte and Doolittle (1982) for a window size of 19 amino-acid residues. The putative membrane-bound domains are indicated by bars. Locations of the amino acid sequence motif, -H-X-X-H-H-, are indicated by arrows.

membrane-bound proteins. Two histidine clusters, which were conserved in the $\Delta 9$ desaturases, are also found in the amino acid sequences of the four desaturases of *Synechocystis* PCC6803 (data not shown). The two histidine clusters located in the hydrophilic regions (Fig. 4-3, indicated by arrows). Histidine residues are included in the iron binding domains in the 18:0-ACP desaturase of castor bean (Fox *et al.* 1993). The coincidence in the partial sequence of the desaturases implies the presence of a common mechanism in the introduction of a double bond into a hydrocarbon chain. It is likely that the two sets of the histidine cluster play an essential role to catalyze the desaturation of fatty acids. Although the role of these histidine residues in the fatty-acid desaturation has not been clarified, one can assume that these residues contribute to the electron transport from ferredoxin to the site of desaturation the fatty acids.

The information of the structure of cyanobacterial desaturases affords a clue to provide answers to questions: how the desaturases catalyze the introduction of a double bond into hydrocarbon chain and how the desaturases identifies the exact position of fatty acids.

The transformant of *E. coli* with the *desC* gene could accumulate 18:1(9), which could not be synthesized in the wild-type cells of *E. coli*. The electron donor of the $\Delta 9$ desaturase in the *E. coli* cells is uncertain. However, it is suggested that the electron donating system in *E. coli* cells could support the reaction of desaturation of 18:0 to 18:1(9) by the $\Delta 9$ desaturase, although *E. coli* cells lack ferredoxin.

Appendices

(A)

AC D13778;
 XX
 DE Synechocystis PCC6714 desA gene encoding delta 12 desaturase.
 XX
 KW delta 12 desaturase.
 XX
 OS Synechocystis PCC6714
 OC Prokaryota; Bacteria; Gracilicutes; Oxyphotobacteria;
 OC Cyanobacteria.
 XX
 RN [1] (bases 1-1176)
 RA Sakamoto T., Wada H., Nishida I., Ohmori M, Murata N.;
 RT "Identification of the conserved domains of delta 12 desaturases
 RT of cyanobacteria.";
 RL Plant Molecular Biology (1994) in press
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(B)

AC D13779;
 XX
 DE *Synechococcus* PCC7002 *desA* gene encoding delta 12 desaturase.
 XX
 KW delta 12 desaturase.
 XX
 OS *Synechocystis* PCC6714
 OC Prokaryota; Bacteria; Gracilicutes; Oxyphotobacteria;
 OC Cyanobacteria.
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 RT "Identification of the conserved domains of delta 12 desaturases
 RT of cyanobacteria.";
 RL Plant Molecular Biology (1994) in press
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(C)

AC D14581;
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 DE Anabaena variabilis desC and desA genes.
 XX
 KW delta 12 desaturase; delta 9 desaturase.
 XX
 OS Anabaena variabilis
 OC Prokaryota; Bacteria; Gracilicutes; Oxyphotobacteria;
 OC Cyanobacteria.
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 RL Plant Molecular Biology (1994) in press
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 RT "Molecular and functional characterization of the delta 9 desaturase
 RT of cyanobacteria.";
 RL (1994) in preparation
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 ** Author requested hold until 01-MAY-1995
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GCTTCCAGCA	CTCTTTTCAG	ATTTTGTCTG	ACCTGAGCGT	CAATTCCAGA	AAAAATCTCT
AACAGTGCCT	GTTCTGCTTG	CCGCAGCTGT	TCCAAGCTGT	TCATTACTTC	CTCGTTAACA
AAAAAACTAT	AAATATGCGA	ATTGCGGAAT	CGCACAGACT	AGGCTGAACA	ATTTTAATTC
GAGTTACTGC	ATGACAATTG	CTACTTCAAC	TAAACCTCAA	ATCAACTGGG	TAAATACCCT
ATTTTTCCTT	GGGCTACACA	TCGGCGCTTT	GTTTGCCTTT	ATCCCTAGTA	ACTTCAGCTG
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(D)

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AC   D13780;
XX
DE   Synechocystis PCC6803 desB gene encoding omega 3 desaturase.
XX
KW   omega 3 desaturase.
XX
OS   Synechocystis PCC6803
OC   Prokaryota; Bacteria; Gracilicutes; Oxyphotobacteria;
OC   Cyanobacteria.
XX
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RA   Sakamoto T., Higashi S, Los DA, Wada H., Nishida I., Ohmori M,
RA   Murata N.;
RT   "The genetic manipulation of membrane lipid unsaturation with
RT   the omega 3 desaturase in cyanobacteria.";
RL   (1994) in preparation
XX
**   Author requested hold until 01-MAY-1995
XX
FH   Key                Location/Qualifiers
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(E)

AC D16547;
 XX
 DE Synechocystis PCC6803 desC gene encoding delta 9 desaturase.
 XX
 KW delta 9 desaturase.
 XX
 OS Synechocystis PCC6803
 OC Prokaryota; Bacteria; Gracilicutes; Oxyphotobacteria;
 OC Cyanobacteria.
 XX
 RN [1] (bases 1-1275)
 RA Sakamoto T., Wada H., Nishida I., Ohmori M, Murata N.;
 RT "Molecular and functional characterization of the delta 9 desaturase
 RT of cyanobacteria.";
 RL (1994) in preparation
 XX
 ** Author requested hold until 01-MAY-1995
 XX
 FH Key Location/Qualifiers
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 FT CDS 79..1035
 FT /gene="desC"
 FT /product="fatty-acid desaturase"
 FT /function="desaturation of fatty acids bound to
 FT glycerolipid at delta 9 position"
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