

Responses to Cold Shock in Cyanobacteria

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Abstract

Acclimation of cyanobacteria to low temperatures involves induction of the expression of several families of genes. Fatty acid desaturases are responsible for maintaining the appropriate fluidity of membranes under stress conditions. RNA-binding proteins, which presumably act analogously to members of the bacterial Csp family of RNA chaperones, are involved in the maintenance of the translation under cold stress. The RNA helicase, whose expression is induced specifically by cold, might be responsible for modifying inappropriate secondary structures of RNAs induced by cold. The cold-inducible family of Clp proteins appears to be involved in the proper folding and processing of proteins. Although genes for cold-inducible proteins in cyanobacteria are heterogeneous, some common features of their untranslated regulatory regions suggest the existence of a common factor(s) that might participate in regulation of the expression of these genes under cold-stress conditions. Studies of the patterns of expression of cold-inducible genes in cyanobacteria have revealed the presence of a cold-sensing mechanism that is associated with their membrane lipids. Available information about cold-shock responses in cyanobacteria and molecular mechanisms of cold acclimation are reviewed in this article.

Introduction

Cyanobacteria are unusual prokaryotic microorganisms that have the ability to perform oxygenic photosynthesis (Margulis, 1975) and they might represent some of the most ancient life forms on earth (Schopf *et al.*, 1965). Cyanobacteria can be found all over the world and in environments from Antarctica, where temperatures never exceed -20°C (Psenner and Sattler, 1998), to hot springs, where temperatures reach 70°C (Ward *et al.*, 1998). Cyanobacteria found in water pockets of Antarctic lake ice, where temperatures are always below 0°C, are metabolically active and capable of performing oxygenic photosynthesis (Prisku *et al.*, 1998). Some species of *Synechococcus* that are characterized by optimum growth temperatures of 55-60°C are also able to fix CO₂ by photosynthesis at these high temperatures (Meeks and Castenholz, 1971). Thus, the cyanobacteria include psychrophilic, psychrotrophic, mesophilic and thermophilic

species that differ from one another with respect to optimal temperatures for growth and the extent of their ability to tolerate temperature stress.

Unicellular and filamentous cyanobacteria have several features that make them particularly suitable for studies of stress responses at the molecular level. The general features of the plasma and thylakoid membranes of cyanobacteria are similar to those of higher-plant chloroplasts in terms of lipid composition and the assembly of proteins. Therefore, cyanobacteria appear to provide a powerful model system for studies of molecular mechanisms of the responses and acclimation of plants to stresses of various kinds (Murata and Wada, 1995; Glatz *et al.*, 1999).

Some strains of cyanobacteria (*Synechocystis* sp. PCC 6803, and *Synechococcus* sp. PCC 7942 and PCC 7002) are naturally able to incorporate foreign DNA that is integrated into the genome by high-frequency homologous recombination (Williams, 1988; Haselkorn, 1991). In other strains, such as the filamentous cyanobacterium *Anabaena* sp. PCC 7120, a method for transformation was developed that is based on the use of plasmids with a broad host-range and bacterial conjugation (Elhai and Wolk, 1988). Since cyanobacteria are characterized by active homologous recombination (Williams and Szalay, 1983; Dolganov and Grossman, 1993), they are widely used in studies of photosynthesis for the production of mutants with disruptions in genes of interest (for review, see Vermaas, 1998).

The complete nucleotide sequence of the genome of *Synechocystis* sp. PCC 6803 has been determined (Kaneko *et al.*, 1996; Kaneko and Tabata, 1997) and the annotated data is now available via the internet (Nakamura *et al.*, 1998). Furthermore, since random mutagenesis of cyanobacteria can be achieved using transposons (Cohen *et al.*, 1994; Schwartz *et al.*, 1998) or antibiotic-resistance cartridges (Labarre *et al.*, 1989), fully sequenced genes can be randomly disrupted and their functions can be examined under certain stress conditions. The complete sequence of the genome facilitates the localization of the sites of mutations and the identification of relevant genes.

Responses of cyanobacterial cells to cold stress are basically of two types. One type involves the cold-induced desaturation of fatty acids in membrane lipids, such that the membranes become less rigid to compensate for the decrease in membrane fluidity that would otherwise occur at the low temperature (Murata and Los, 1997). The other type involves the cold-induced synthesis of enzymes that enhance the efficiency of transcription and translation to compensate for the decrease in the efficiency of these processes that would otherwise occur at the low temperature (Sato, 1994, 1995). Both types of response serve to protect the cyanobacteria from the detrimental effects of cold stress.

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Cold-Inducible Genes and their Regulation in Cyanobacteria

The families of cold-inducible genes in cyanobacteria that have been reported to date are listed in Table 1. The first cold-inducible genes to be characterized in cyanobacteria were genes for fatty acid desaturases (Wada *et al.*, 1990; Murata and Wada, 1995). The fatty acid desaturases maintain the appropriate physical state of the cell membranes (Murata and Los, 1997). Subsequently, genes for RNA-binding proteins (Rbps) were identified as cold-inducible genes (Sato, 1995). The Rbps appear to act similarly to the Csp RNA chaperones of *Escherichia coli* and *Bacillus subtilis*. The S21 protein in the small subunit of ribosomes was also shown to be induced by cold and to accumulate transiently in ribosomes at low temperatures (Sato, 1994). Next, Clp proteins were discovered as a novel family of cold-shock chaperones and proteases (Celerin *et al.*, 1998). Most recently, genes for RNA helicases (Chamont *et al.*, 1999), which appear to establish the appropriate secondary structure of mRNAs, were identified as being inducible by cold.

Desaturases

Acyl-Lipid Desaturases

There are three types of fatty acid desaturase and the acyl-lipid desaturases are one such type (Murata and Wada, 1995). They are characterized by their ability to convert a single bond in a fatty acyl chain, which has been esterified to a membrane glycerolipid, into a double bond. In other

words, they catalyze the introduction of individual double bonds (Sato *et al.*, 1979; Sato and Murata, 1980; Murata and Wada, 1995). The desaturation of fatty acids and the expression of genes for desaturase have been extensively studied in *Synechocystis* sp. PCC 6803. This cyanobacterium has four acyl-lipid desaturases (Figure 1A), which catalyze desaturation at the $\Delta 9$, $\Delta 12$, $\Delta 6$ and $\omega 3$ positions, respectively, of fatty acids that are located at the *sn*-1 position of the glycerol moieties of glycerolipids (Murata *et al.*, 1992; Higashi and Murata, 1993). The $\Delta 9$ desaturase introduces the first unsaturated bond into stearic acid to produce oleic acid, which is further desaturated to linoleic acid by the $\Delta 12$ desaturase. The $\Delta 6$ and $\omega 3$ desaturases then introduce unsaturated bonds to generate tri- and tetraunsaturated fatty acids (Figure 1A). By contrast to *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7942 has only one gene for a desaturase, namely, the *desC* gene for the $\Delta 9$ desaturase. Thus, the cells contain saturated and monounsaturated fatty acids but no polyunsaturated fatty acids (Figure 1B).

Expression of the Genes for Fatty Acid Desaturases in *Synechocystis* sp. PCC 6803

The *desA* gene for the $\Delta 12$ desaturase in *Synechocystis* sp. PCC 6803 was the first gene for an acyl-lipid desaturase to be cloned (Wada *et al.*, 1990), and the low temperature-dependent expression of this gene has been studied extensively (Los *et al.*, 1993; 1997). The level of the transcript increases 10-fold within 30 min of a downward shift in temperature from 34°C to 22°C. The accumulation

Table 1. Genes Known to be Induced by Cold Stress in Cyanobacteria

Gene	Gene product	Cyanobacterium	Reference
Desaturase family			
<i>desA</i>	$\Delta 12$ desaturase	<i>Synechocystis</i> sp. PCC 6803 <i>Synechocystis</i> sp. PCC 6714 <i>Synechococcus</i> sp. PCC 7002 <i>Spirulina platensis</i>	Wada <i>et al.</i> , 1990 Sakamoto <i>et al.</i> , 1994a Sakamoto and Bryant, 1997 Murata <i>et al.</i> , 1996
<i>desB</i>	$\omega 3$ desaturase	<i>Synechocystis</i> sp. PCC 6803 <i>Synechococcus</i> sp. PCC 7002	Sakamoto <i>et al.</i> , 1994c Sakamoto and Bryant, 1997
<i>desC</i>	$\Delta 9$ desaturase	<i>Synechococcus</i> sp. PCC 7942 <i>Synechococcus</i> sp. PCC 7002	Ishizaki-Nishizawa <i>et al.</i> , 1996 Sakamoto and Bryant, 1997
<i>desD</i>	$\Delta 6$ desaturase	<i>Synechocystis</i> sp. PCC 6803 <i>Spirulina platensis</i>	Reddy <i>et al.</i> , 1994 Murata <i>et al.</i> , 1996
Rbp family			
<i>rbpA1</i>	RNA-binding protein (RbpA1)	<i>Anabaena variabilis</i> M3	Sato and Nakamura, 1998
<i>rbpA2</i>	RNA-binding protein (RbpA2)	<i>Anabaena variabilis</i> M3	Sato, 1995
<i>rbpA3</i>	RNA-binding protein (RbpA3)	<i>Anabaena variabilis</i> M3	Sato and Maruyama, 1997
<i>rbpC</i>	RNA-binding protein (RbpC)	<i>Anabaena variabilis</i> M3	Sato, 1995
RNA helicases			
<i>crhB</i>	RNA helicase (CrhB)	<i>Anabaena</i> sp. PCC 7120	Chamot <i>et al.</i> , 1999
<i>crhC</i>	RNA helicase (CrhC)	<i>Anabaena</i> sp. PCC 7120	Chamot <i>et al.</i> , 1999
Clp family			
<i>clpB</i>	Molecular chaperone (ClpB)	<i>Synechococcus</i> sp. PCC 7942	Porankiewicz and Clarke, 1997
<i>clpP1</i>	Protease (ClpP)	<i>Synechococcus</i> sp. PCC 7942	Porankiewicz <i>et al.</i> , 1998
<i>clpX</i>	Unknown	<i>Synechococcus</i> sp. PCC 7942	Porankiewicz <i>et al.</i> , 1998
Others			
<i>rpsU</i>	Ribosomal subunit (S21)	<i>Anabaena variabilis</i> M3	Sato, 1994
<i>lti2</i>	Unknown (Lti2)	<i>Anabaena variabilis</i> M3	Sato, 1992

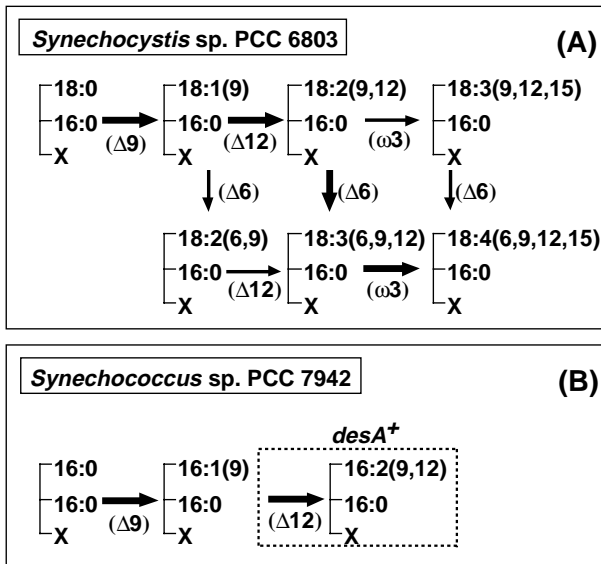


Figure 1. Schematic Representation of the Desaturation of Fatty Acids in the Membrane Lipids of two Species of Cyanobacteria. Glycerolipids esterified exclusively with saturated fatty acids are synthesized first. Then the fatty acids are desaturated to yield their monounsaturated derivatives by acyl-lipid $\Delta 9$ desaturase and these derivatives are converted to polyunsaturated fatty acids by other acyl-lipid desaturases (Sato and Murata, 1980; Higashi and Murata, 1993; Murata and Wada, 1995). (A) *Synechocystis* sp. PCC 6803. Thick and thin arrows indicate major and minor pathways, respectively. (B) *Synechococcus* sp. PCC 7942. The reaction enclosed by a dotted line has been introduced by transformation with the *desA* gene for the $\Delta 12$ desaturase from *Synechocystis* sp. PCC 6803. X represents the polar head group. Adapted from Murata *et al.* (1992).

of the transcript is caused by both the activation of transcription and the enhanced stabilization of the mRNA at low temperature (Los and Murata, 1994; Los *et al.*, 1997). When the temperature returns to normal, the *desA* mRNA rapidly disappears (Los *et al.*, 1997).

The *desD* gene for the $\Delta 6$ desaturase is also induced by a downward shift in temperature, as demonstrated by Northern and Western blotting analyses (Los *et al.*, 1997). The level of *desD* mRNA increases about 10-fold within 15 min, while the level of the enzyme itself doubles within 4 h of the start of cold treatment.

Of the three cold-inducible genes for desaturases in *Synechocystis* sp. PCC 6803, it is the *desB* gene for the $\omega 3$ desaturase that responds most dramatically to a decrease in temperature: The level of *desB* mRNA increases 15-fold within 10 min after a shift in temperature from 34°C to 22°C (Los *et al.*, 1997). The accumulation of *desB* mRNA due to both the acceleration of transcription and the stabilization of the transcript. Figure 2 shows the kinetics of accumulation of *desB* mRNA, of the $\omega 3$ desaturase, and of $\omega 3$ -unsaturated fatty acids [α -linolenic (α -18:3) and stearidonic (18:4) acids] in *Synechocystis* sp. PCC 6803 after a shift in temperature from 35°C to 25°C. As shown in Figure 2, *desB* mRNA accumulates rapidly and then its level starts to decline gradually. The $\omega 3$ desaturase is barely detectable at 35°C but its level becomes gradually higher as time passes after the shift in temperature. Then, the level of the $\omega 3$ desaturase remains high for 10 h (Figure 2). The accumulation of the $\omega 3$ desaturase is followed by the slow and gradual accumulation of $\omega 3$ -unsaturated fatty acids.

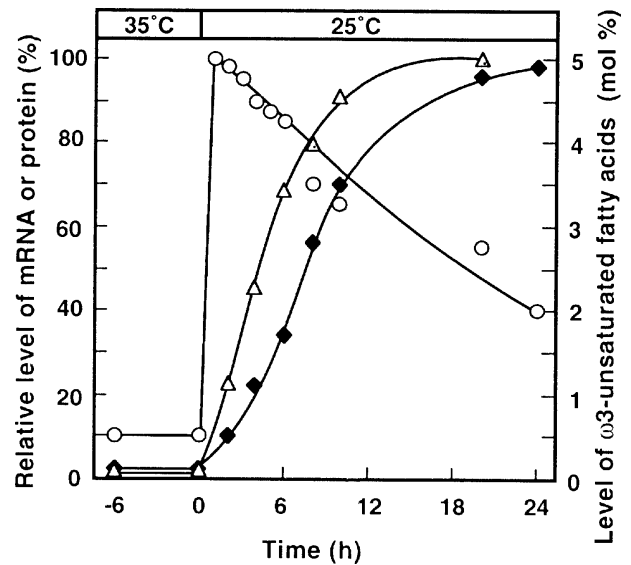


Figure 2. Changes in *Synechocystis* sp. PCC 6803 in levels of the transcript of *desB*, the encoded $\omega 3$ desaturase and $\omega 3$ -unsaturated fatty acids after a shift in temperature from 35°C to 25°C. O-O, *desB* mRNA; Δ - Δ , $\omega 3$ desaturase; \blacksquare - \blacksquare , $\omega 3$ -unsaturated fatty acids (*i.e.*, the level of α -18:3 plus 18:4 relative to the total fatty acids).

Primer extension analysis indicated that transcription of the *desA*, *desB*, and *desD* genes starts at positions -114, -35, and -347, respectively, counted from the site of transcription start, at both 34°C and 22°C. Thus, it appears that RNA polymerase utilizes the same promoters at both temperatures. An alignment of nucleotide sequences near the sites of initiation of transcription of the genes for the cold-inducible desaturases revealed a consensus sequence, GTTTGTTTT, just downstream of these sites, irrespective of the position of each initiation site (see also Section 4).

Although expression of genes for desaturases has been studied most extensively in *Synechocystis* sp. PCC 6803, there are several reports of the cold-induced expression of genes for desaturases in other species of cyanobacteria (Table 1). The *desC* gene in *Synechococcus* sp. PCC 7002 is cold-inducible and the level of its transcript increases markedly within 15 min of a shift from 38°C to 22°C (Sakamoto and Bryant, 1997). The *desA* and *desB* genes are also induced rapidly in this cyanobacterium (Sakamoto *et al.*, 1997). In *Synechococcus* sp. PCC 7942, which only has a *desC* gene for the $\Delta 9$ desaturase (Figure 1B), the gene is induced 30 min after a shift from 36°C to 24°C (Ishizaki-Nishizawa *et al.*, 1996).

Biological Functions of Fatty Acid Desaturases

The importance of the desaturases and of the expression of their genes in the acclimation to cold of cyanobacteria has been well documented (Wada *et al.*, 1990; Gombos *et al.*, 1992; 1994; Wada *et al.*, 1994; Tasaka *et al.*, 1996; for reviews, see Murata and Wada, 1995; Los and Murata, 1998). A series of mutants of *Synechocystis* sp. PCC 6803, which are defective in a stepwise manner in the desaturation of fatty acids in membrane lipids, was generated by targeted mutagenesis of individual desaturases (Tasaka *et al.*, 1996). Targeted mutagenesis

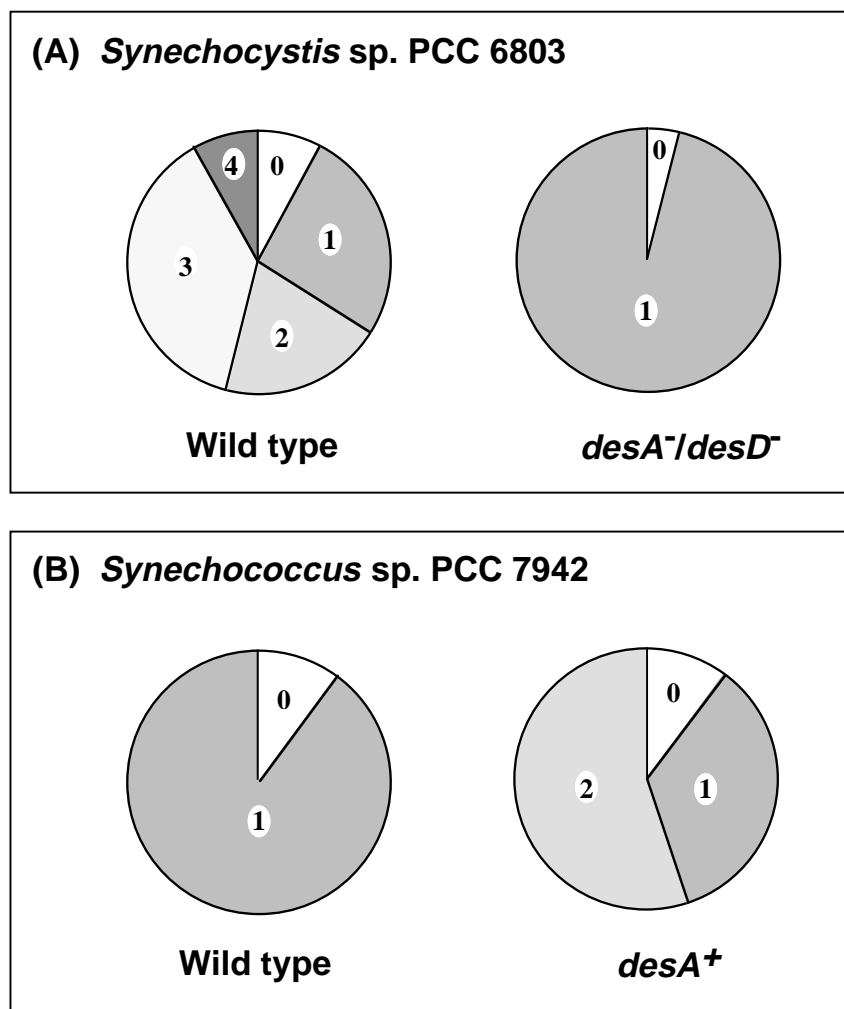


Figure 3. Changes in the Unsaturation of Fatty Acids in Cyanobacterial Cells by Genetic Manipulation of Acyl-Lipid Desaturases

(A) The fatty acid composition of glycerolipids in wild-type and *desA⁻/desD⁻* cells of *Synechocystis* sp. PCC 6803 after growth at 25°C. (B) The fatty acid composition of glycerolipids in wild-type and *desA⁺* cells of *Synechococcus* sp. PCC 7942 after growth at 25°C. Numbers in the pie charts represent the numbers of double bonds in the individual molecular species of lipids. Adapted from Tasaka *et al.* (1996) and Los and Murata (1998).

of both the *desA* gene for the $\Delta 12$ desaturase and *desD* gene for the $\Delta 6$ desaturase resulted in dramatic changes in the fatty acids in membrane lipids. There was a considerable increase in the level of monounsaturated oleic acid at the expense of polyunsaturated fatty acids, such as di-, tri-, and tetraunsaturated fatty acids (Figure 3A). Cells with these two mutations grew as well as wild-type cells at 35°C but did not grow well at 25°C. At 20°C, the *desA⁻/desD⁻* mutant cells were unable to propagate, whereas wild-type cells grew relatively well at this temperature (Tasaka *et al.*, 1996). Moreover, *desA⁻/desD⁻* cells failed to recover from photo-induced damage to the photosystem II complex at low temperatures. Apparently, they were unable to process the precursor to the D1 protein to generate the mature D1 protein, an essential component of the reaction center of the photosystem II complex (Kanervo *et al.*, 1997). Since only two genes for fatty acid desaturases were inactivated in the *desA⁻/desD⁻* strain, we can reasonably conclude that the ability of *Synechocystis* sp. PCC 6803 to tolerate cold stress is determined by the presence of polyunsaturated fatty acids.

A similar conclusion is reached with another set of experiments that involved transformation of *Synechococcus* sp. PCC 7942, which normally contains only saturated and monounsaturated fatty acids (Figure 1B), with the *desA* gene for the $\Delta 12$ desaturase of

Synechocystis sp. PCC 6803 (Figure 3B). The transformed *desA⁺* cells synthesized diunsaturated fatty acids at the expense of monounsaturated fatty acids, and they were able to tolerate lower temperatures than wild-type cells (Wada *et al.*, 1990; 1994). Moreover, the *desA⁺* cells appeared to be more tolerant to photoinhibition (Gombos *et al.*, 1997). During cold acclimation, wild-type cells of *Synechococcus* sp. PCC 7942 replace one isoform of the D1 protein (D1:1) with another isoform (D1:2) within a few hours (Campbell *et al.*, 1995). This replacement appears to be essential for the survival of this cyanobacterium at low temperatures. The transformation with the *desA* gene shifted the temperature critical for the replacement of the D1:1 form with the D1:2 form toward a lower temperature (Sippola *et al.*, 1998). Although the molecular mechanism for the shift in the critical temperature is not known, these observations reveal that the desaturation of fatty acids in membrane lipids is an important factor in the acclimation to cold.

The Rbp Family

Rbps (RNA binding proteins) are involved in various aspects of the metabolism of RNA, such as splicing, modification, maintenance of stability and translation (Kenan *et al.*, 1991; Nagai *et al.*, 1995). The Rbps in the chloroplasts of higher plants contain two RNA-recognition

motifs, an amino-terminal acidic domain and a carboxy-terminal glycine-rich domain (Ye *et al.*, 1991). Sato (1995) characterized the family of cold-inducible genes that encode Rbps in *Anabaena variabilis* strain M3. The Rbps of this cyanobacterium can be divided into those with a glycine-rich carboxy-terminal domain and those without such a domain.

Sato (1995) demonstrated that, among eight *rbp* genes found in *Anabaena variabilis*, four genes (*rbpA1*, *rbpA2*, *rbpA3*, *rbpB* and *rbpC*) for proteins, that contain glycine-rich domains are regulated by cold. Two genes (*rbpB* and *rbpD*) for proteins without glycine-rich domains are expressed more or less constitutively (Sato, 1995; Sato and Maruyama, 1997). The mRNAs for *rbpA1* and *rbpA2* are barely detectable at 38°C. *RbpA1* mRNA becomes easily detectable 30 min after cells have been transferred from 38°C to 22°C. The level of this mRNA reaches a maximum within 3 h and then gradually decreases (Sato, 1995). Expression of *rbpA2* is induced within 10 min after transfer from 38°C to 22°C. The level of its transcript reaches a maximum in 2 h, and then declines gradually. *RbpC* mRNA is detectable at 38°C, and its level increases about 10-fold within 1 h after transfer from 38°C to 22°C (Sato, 1995). The levels of the corresponding proteins also increase dramatically after cold shock. However, whereas levels of transcripts of the cold-inducible *rbp* genes increase transiently, the levels of the corresponding proteins increase gradually and remain maximal for 24 h after the shift in temperature from 38°C to 22°C (Sato, 1995).

Sato and Maruyama (1997) demonstrated that transcription of the *rbpA3* gene is driven by two different promoters. One of the promoters is active at high temperatures and its activity is suppressed at low temperatures. By contrast, the activity of the other promoter increases transiently after a shift in temperature from 38°C to 22°C. Using a *lacZ* reporter fused to a number of modified promoter regions, Sato and Nakamura identified a putative cold-responsive *cis*-acting element in a 5'-untranslated region (5'-UTR) of the *rbpA1* gene (Sato and Nakamura, 1998). A 150-bp region of DNA, which is located between the site of initiation of transcription and a ribosome-binding site, is absolutely necessary for the cold-induced transcription of the *rbpA1* gene. Deletions within this region result in constitutive transcription at both 38°C and 22°C (Sato and Nakamura, 1998). This observation suggests that transcription of the *rbpA1* gene might be repressed at high temperatures by an unidentified repressor protein. The argument in favor of the existence of such a protein is strengthened by the results of gel mobility shift assays with the target DNA sequence as a probe and a crude extract of proteins from cells grown at a high temperature. A protein(s) that bound to the 5'-UTR of the *rbpA1* gene was present in the crude extract of cells grown at 38°C but not of cells grown at 22°C. Affinity purification of DNA-binding proteins demonstrated the presence of two putative repressor proteins of about 75 kDa and about 32 kDa, respectively (Sato and Nakamura, 1998).

The discovery of cyanobacterial Rbps whose synthesis is regulated by temperature revealed the existence of a new class of stress-inducible RNA-binding proteins. The sequence of the genome of *Synechocystis* sp. PCC 6803 indicates that cyanobacteria do not have the cold-shock proteins (Csps) that are found in some eubacteria (Jones and Inoue, 1994). The synthesis of Csps is rapidly induced

by cold and Csps are thought to play important roles in the regulation of transcription under cold stress in *E. coli* and *B. subtilis* (Jones and Inoue, 1994; Schnuchel *et al.*, 1993). Some Csps, known as RNA chaperones, bind to single-stranded DNA and RNA *in vitro* with a molecular surface that corresponds to consisting of a β -sheet, a phenomenon that is analogous to the binding of RNA-recognition motifs to RNAs (Thieringer *et al.*, 1998). Both the Rbps in cyanobacteria and the Csps in *E. coli* and *B. subtilis* are induced by cold and bind RNA. Therefore, it is possible that Rbps might function similarly to the Csps.

Cold-Induced RNA Helicases

RNA helicases are responsible for modifying the secondary structure of mRNAs, which is a critical factor in the regulation of translation (Fuller-Pace, 1994). RNA helicases in *E. coli* play important roles in the assembly of ribosomes (Nishi *et al.*, 1988; Nicol and Fuller-Pace, 1995), the turnover of RNA (Py *et al.*, 1996), and the acclimation to cold (Jones *et al.*, 1996). Two genes for RNA helicases, *crhB* and *crhC*, have been identified in *Anabaena* sp. PCC 7120 (Chamot *et al.*, 1999). The *crhB* gene is expressed under variety of stress conditions (*e.g.*, cold stress, salt stress, nitrogen limitation), while expression of the *crhC* gene occurs exclusively in response to cold stress. The *crhC* gene is expressed specifically in cells that have been transferred from 30°C to 20°C, with the level of *crhC* mRNA increasing more than 100-fold during incubation of cells at 20°C for 3 h (Chamot *et al.*, 1999).

The deduced amino acid sequence of the CrhC protein led to its identification as a novel RNA helicase that belongs to the DEAD (Asp-Glu-Ala-Asp) box family of helicases (Gorbalenya and Koonin, 1993). However, CrhC has a novel FAT (Phe-Ala-Thr) box instead of the canonical SAT (Ser-Ala-Thr) box that is characteristic of known DEAD box RNA helicases (Py *et al.*, 1996). The suggested role of CrhC in cold acclimation is the destabilization of the secondary structures of mRNAs, which allows cells to overcome the cold-induced blockage of the initiation of translation that occurs at low temperatures (Chamot *et al.*, 1999).

Protein S21, a Component of the Small Subunit of Ribosomes

Cyanobacterial ribosomes are of the prokaryotic type and are similar to those in *E. coli* (Gray and Herson, 1976; Sato *et al.*, 1998). The *rpsU* gene for protein S21, a component of the small subunit of ribosomes, is located just downstream of the *rbpA1* gene in the genome of *Anabaena variabilis* strain M3 (Sato, 1994). Two transcripts are characteristic of this gene cluster. The level of the combined transcripts of the *rbpA1* and *rpsU* genes increases 10-fold within 2.5 h of a shift from 38°C to 22°C. By contrast, the minor monocistronic transcript of *rpsU* is more abundant at 38°C (Sato, 1994). The level of the S21 protein increases 3-fold after the shift in temperature. Sato *et al.* (1997) demonstrated that, in isolated ribosomes, the S21 protein is present at an equimolar level relative to other ribosomal proteins at 22°C, but the relative level of S21 decreases at high temperatures.

A cold-induced increase in the level of S21 protein has also been found in *Synechocystis* sp. PCC 6803, in which the *rpsU* gene is not adjacent to the *rbpA* gene but is located downstream of the rRNA operon (Sato *et al.*, 1997).

	Gene	Source	Reference
TGGCAACGTGTTATAAAAAAGAAA <u>G</u> TTTG- <u>TTT</u> ACCTG	<i>desA</i>	6803	(Los et al., 1997)
GCCTTCTTTTAGGATAGAATCATAG <u>G</u> ATTG- <u>TTTT</u> GCCG	<i>desB</i>	6803	(Los et al., 1997)
TAGCAAATAAGTTTAATTCATAA <u>C</u> TGAG- <u>TTTT</u> ActG	<i>desD</i>	6803	(Los et al., 1997)
<u>TCCGAAATTTACATCTCTAGACAGTAACAATTTTG</u>	<i>rbpA1</i>	A.v.	(Sato and Nakamura, 1998)
TCCGAAACCTAAATCTCTACGTACCTATGATTTCCG	<i>rbpA2</i>	A.v.	(Sato and Nakamura, 1998)
TCCGAAATTTAAATCTCTACACATTTATGATTTTG	<i>rbpA3</i>	A.v.	(Sato and Maruyama, 1997)
TCCGAAATCTCAATCCCTAGACACTTCTGATTTTG	<i>rbpB</i>	A.v.	(Sato and Nakamura, 1998)
CTTACCATTATGAGCCATTAATTAAGCTAATTTAGCAG	<i>rpsU</i>	A.v.	(Sato et al., 1997)
GCTTAATACTAGCATTFTTATATTTTACTGATTTT	<i>lti2</i>	A.v.	(Sato, 1992)
TCCnAAATTTAPATnnATAnATAnnTnTGATTTTGcNg	CONSENSUS		

Figure 4. Alignment of the 5'-Untranslated Regions of Various Cold-Inducible Genes from Cyanobacteria. The sites of initiation of transcription of the genes for desaturases are double-underlined. Part of the 5'-UTR of the *rbpA1* gene to which a *trans*-acting protein factor(s) binds is underlined. See text for further details. Abbreviations: A.v., *Anabaena variabilis* strain M3; 6803, *Synechocystis* sp. PCC 6803.

The changes in level of S21 protein in cyanobacterial ribosomes with changes in temperature raise an interesting question about the role of this protein in the acclimation of the translational apparatus to cold stress. It is possible that a ribosome without S21 protein might be inactive under cold conditions and that it is only when S21 is present that the ribosome becomes translationally active. The pattern of the cold-induced accumulation of S21 in cyanobacteria suggests that this protein might be involved in the acclimation to cold of the translational apparatus, whose activity appears always to decrease in prokaryotes upon exposure to cold shock.

In *E. coli*, S21 is a constitutive component of the ribosome. It is likely that nonphotosynthetic bacteria express the *rpsU* gene constitutively and that S21 is always present in ribosomes at an appropriate stoichiometric level (Held *et al.*, 1974). However, the *rpsU* gene is absent from the genome of the parasitic prokaryote *Mycoplasma genitalium* (Frazer *et al.*, 1995) and S21 is absent from chloroplast ribosomes (Harris *et al.*, 1994), as well as from cytoplasmic ribosomes (Sato *et al.*, 1997), in higher plants.

The Clp Family

Caseinolytic proteases (Clps) represent a new family of bacterial molecular chaperones that includes proteases that are expressed constitutively in some cases and stress-inducibly in others (Schirmer *et al.*, 1996; Thompson and Maurizi, 1994; Kessel *et al.*, 1995). The sequence of the genome of *Synechocystis* sp. PCC 6803 (Kaneko *et al.*, 1996) indicates that it contains genes for ClpB, ClpC, ClpP, and ClpX. Moreover, up to four isozymes of ClpP are encoded by a multigene sub-family.

In *Synechococcus* sp. PCC 7942, the *clpP1* gene is found within the *clpP1/clpX* operon (Porankiewicz *et al.*, 1998). Rapid accumulation of ClpP1 is observed under cold stress and also under UV-B light, and the amount of ClpP1 increases 15-fold within 24 h of the start of cold treatment (Porankiewicz *et al.*, 1998).

Growth of a *clpP1* null mutant, $\Delta clpP1$, is severely inhibited at low temperatures. Wild-type cells survive and

propagate after a shift from 37°C to 25°C, while $\Delta clpP1$ mutant cells completely lose viability at 25°C (Porankiewicz and Clarke, 1997). During cold acclimation, wild-type cells replace one isoform of the D1 protein (D1:1) with another isoform (D1:2) within just a few hours. Once acclimated to low temperatures, wild-type cells then replace the D1:2 isoform by the D1:1 isoform. By contrast, $\Delta clpP1$ cells fail to perform this final step (Porankiewicz *et al.*, 1998). Although the mechanism responsible for the exchange of the D1:1 and D1:2 isoforms in this cyanobacterium is poorly understood, such observations demonstrate that ClpP1 is indispensable to the acclimation to cold. It is of particular interest in this context that the ClpP protein exhibits peptidase activity in *E. coli* (Maurizi *et al.*, 1990a,b). ClpP1 in *Synechococcus* sp. PCC 7942 might participate in the exchange of isoforms of the D1 protein by degrading the inappropriate isoform of this protein.

ClpB (HSP100) in *Synechococcus* sp. PCC 7942 was defined initially as a heat-inducible molecular chaperone that is essential for the acquisition of thermotolerance (Eriksson and Clarke, 1996). However, synthesis of ClpB is also strongly induced under cold stress (Porankiewicz and Clarke, 1997; Celerin *et al.*, 1998). Targeted mutagenesis of the *clpB* gene accelerated inhibition of the activity of the photosystem II complex at low temperatures and reduced the ability of mutant cells to acclimate to low temperatures in terms of propagation and survival. Porankiewicz and Clarke (1997) suggested that ClpB might renature and solubilize aggregated proteins at low temperatures at which translation is repressed.

Expression of the *lti2* Gene is Induced by Low Temperature

A low temperature-inducible gene, *lti2*, was cloned from the genome of *Anabaena variabilis* strain M3 (Sato, 1992). This gene exhibits significant homology to genes for various α -amylases and to genes for glucanotransferases of bacteria, fungi, and higher plants. The Lti2 protein expressed *in vitro* has no α -amylase activity (Sato, 1992). Sato suggested that the Lti2 protein might be a kind of

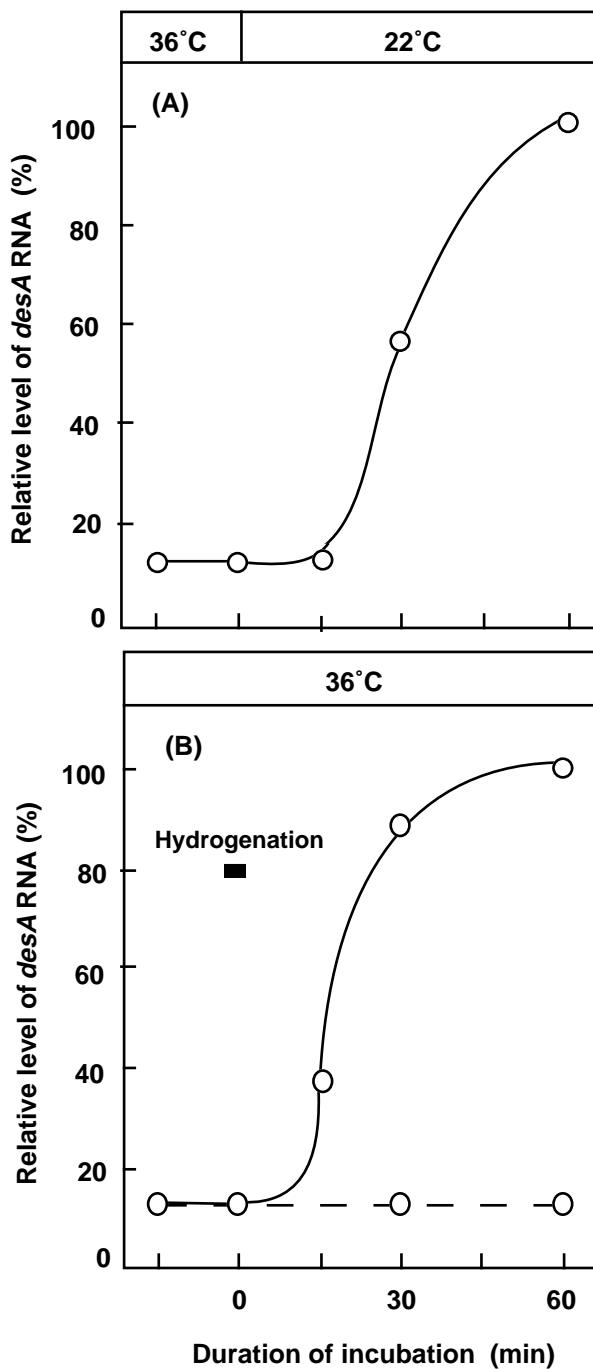


Figure 5. Changes in the Level of the *desA* Transcript in *Synechocystis* sp. PCC 6803

(A) Temperature-induced accumulation of *desA* mRNA. Cells that had been grown at 36°C were transferred to 22°C at time zero. (B) Hydrogenation-induced accumulation of *desA* mRNA. Cells that had been grown at 36°C were subjected to chemical hydrogenation at 36°C for 4 min (indicated by a horizontal bar). Under these conditions, 5% of the fatty acids in the glycerolipids in the plasma membrane were hydrogenated while practically no lipids in the thylakoid membranes were hydrogenated. Adapted from Los *et al.* (1993) and Vigh *et al.* (1993).

glucanotransferase since it contains regions of conserved amino acids that are characteristic of the catalytic centers of glucanotransferases (Sato, 1992).

The level of the transcript of the *lti2* gene increases 40-fold within one hour of a shift from 38°C to 22°C.

Moreover, a homolog of the *lti2* gene in *Anabaena* sp. PCC 7120 responds to high-salt stress and to osmotic stress in addition to low-temperature stress (Schwartz *et al.*, 1998). An *orrA* (osmotic response regulator) gene for the regulator of the response of the *lti2* gene to salt stress has been identified in *Anabaena* sp. PCC 7120. The *orrA* gene is homologous to a regulator of the expression of a gene for an extracellular proteinase in *B. subtilis* (Kunst *et al.*, 1997). Thus, it has been suggested that a two-component regulatory system exists to control expression of the *lti2* gene in response to changes in osmotic pressure (Schwartz *et al.*, 1998). The existence of a cold-response regulator of expression of the *lti2* gene and the biological role of the Lti2 protein under stress conditions remain to be established.

Some Common Features of the Regulatory Regions of Cold-Inducible Genes

An alignment of the 5'-untranslated regions (5'-UTRs) of various cyanobacterial genes whose expression is induced by cold reveals some common sequence (Figure 4). The consensus sequence corresponds to part of the 5'-UTR of the *rbpA1* gene of *A. variabilis* to which a *trans*-acting protein factor(s) binds (Sato and Nakamura, 1998). Expression of the *rbpA1* gene is suppressed at high temperatures by this interaction (Sato and Nakamura, 1998). Expression of the *desB* gene for the ω 3 desaturase in *Synechocystis* sp. PCC 6803 seems to be suppressed at high temperatures by the interaction of a large acidic protein with the region that includes the consensus sequence (our unpublished results). It remains to be determined whether the putative repressors of all the cold-inducible genes are identical or even similar.

Membrane Fluidity as a Link Between the Temperature of the Environment and the Induction of Gene Expression

The expression of the various above-mentioned genes is induced at low temperatures or after a downward shift in temperature but it remains unclear how cyanobacterial cells detect the ambient temperature or a change in the ambient temperature that leads to the cold-induced expression of genes. The most extensive study of this problem to date has involved expression of the genes for desaturases and its relationship to the dynamics of membrane structure in *Synechocystis* sp. PCC 6803. It is important in this context to recall here that the extent of unsaturation of the fatty acids in membrane lipids is the major factor that determines the fluidity of the membrane (Kates *et al.*, 1984; Cossins, 1994). Several lines of evidence exist for the contribution of either membrane fluidity or the unsaturation of fatty acids in membrane lipids to the perception of the temperature signal, as follows.

1) We compared the temperature-dependent expression of the cold-inducible *desA* gene in two types of cell that had been grown at different temperatures. Fatty acids in cells grown at 32°C were more unsaturated than those in cells grown at 36°C, an indication that membranes in cells grown 32°C were more fluid than those in cells grown at 36°C. In cells grown at 36°C, the cold-induced expression of the *desA* gene began to appear when the temperature was lowered to 28°C, whereas in cells grown

at 32°C it began to appear at 26°C (Los *et al.*, 1993). These observations indicated that, in these cyanobacterial cells, an increase in unsaturation of fatty acids in membrane lipids and, thus, in the fluidity of membranes, shifted the cold-induced expression of the *desA* gene toward a lower temperature. It was clear that cyanobacterial cells perceived a change in temperature and not the absolute temperature and, moreover, that the cells sensed a change in temperature only when it exceeded 6°C. In terms of the physical response, this biological perception of temperature is very sensitive since a decrease in temperature of 6°C corresponds to a reduction in the molecular motion in membrane lipids of only 2% (Murata and Los, 1997).

2) We have been able to decrease the fluidity of membranes by the catalytic hydrogenation, under isothermal conditions, of the fatty acids of the lipids in the plasma membrane of *Synechocystis* sp. PCC 6803 (Vigh *et al.*, 1993). This chemical method allowed us to examine the direct effects of membrane fluidity in the presence of only minimal contributions by other changes caused by a change in temperature. Hydrogenation of cells at 36°C for 4 min converted 5% of the unsaturated fatty acids to saturated fatty acids in the glycerolipids of plasma membranes, but not of thylakoid membranes. The decrease in membrane fluidity, caused either by cold stress or by catalytic hydrogenation, resulted in rapid activation of the expression of the *desA* gene (Figure 5). Moreover, hydrogenation of lipids in plasma membranes increased the threshold temperature for the expression of the *desA* gene from 28°C to 30°C (Vigh *et al.*, 1993). These findings suggest that the primary signal in the perception of temperature is a change in the fluidity of the plasma membrane.

3) We compared the temperature-dependent expression of the cold-inducible *desB* gene in wild-type and *desA⁻/desD⁻* cells of *Synechocystis* sp. PCC 6803 (Figure 3A). In wild-type cells that had been grown at 36°C and contained mono-, di-, and triunsaturated fatty acids, transcription of the *desB* gene was induced at 28°C. By contrast, in *desA⁻/desD⁻* cells that had been grown at 36°C and contained monounsaturated but no polyunsaturated fatty acids, transcription of the *desB* gene was induced at 32°C (our unpublished results). These results suggest that the replacement of polyunsaturated by monounsaturated fatty acids and, therefore, a decrease in membrane fluidity, shifted the cold-induced expression of the *desB* gene toward lower temperatures by 5°C in *desA⁻/desD⁻* cells.

4) We also compared the temperature-dependent expression of *desC*, the cold-inducible gene for the $\Delta 9$ desaturase, in wild-type and *desA⁺* cells of *Synechococcus* sp. PCC 7942 (Figure 3B). In wild-type cells that produced monounsaturated but no polyunsaturated fatty acids, induction of expression of the *desC* gene occurred at 30°C. In *desA⁺* cells that produced diunsaturated fatty acids at the expense of monounsaturated fatty acids, induction of the expression of the *desC* gene occurred at 28°C (our unpublished results). These observations indicated that, in *desA⁺* cells, replacement of most of the monounsaturated fatty acids by diunsaturated fatty acids and, therefore, an increase in membrane fluidity shifted the cold-induced expression of the *desC* gene toward higher temperatures by 2°C.

The discovery of an apparent feedback link between membrane fluidity and expression of genes for desaturases

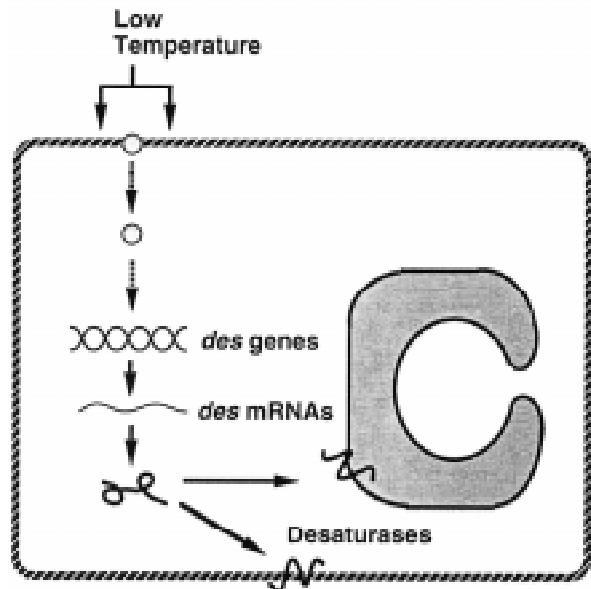


Figure 6. Model of the Induction of Desaturases in Cyanobacterial Cells. Proposed model of the perception of temperature and transduction of the signal during the low temperature-induced expression of genes for desaturases in cyanobacterial cells. Putative sensor and signal-transducing components are indicated by circles and dotted arrows, respectively. PM, Plasma membrane; TM, thylakoid membrane; *des*, desaturase; Pre, precursor to desaturase.

suggests that a temperature sensor might be located in the cyanobacterial plasma membrane that perceives changes in membrane fluidity and transmits the signal to activate the expression of the genes for desaturases (Murata and Wada, 1995; Murata and Los, 1997). The putative chain of events from cold shock to the induction of the genes for desaturases is summarized schematically in Figure 6. The pathway from the perception of temperature to the induction of the genes for desaturases is now being investigated in our laboratory. Subsequent reactions have been well characterized. After a downward shift in temperature or a decrease in membrane fluidity, the expression of the genes for desaturases is enhanced and the desaturases are synthesized *de novo* and targeted to both the plasma membrane and the thylakoid membranes (Mustardy *et al.*, 1996). These enzymes catalyze the desaturation of the fatty acids in the membrane lipids to compensate for the decrease in membrane fluidity that has been caused by cold stress.

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