

**Genetically Engineered Alteration of
Stress Tolerance in *Synechococcus*:
Protective Roles of Glycinebetaine**

Patcharaporn Deshniem

DOCTOR OF SCIENCE

Department of Molecular Biomechanics
School of Life Science
The Graduate University for Advanced Studies

1995

Contents

	Page
Acknowledgments	3
Abstract	4
Chapter 1 General Introduction	5
Response of the cell to environmental stresses	6
1.1. Responses to salt stress	6
1.1.1. Induction of ion-transport activity	7
1.1.2. Enhancement of respiration	11
1.1.3. Changes in ultrastructure and membrane-lipid composition	12
1.1.4. Induction of salt-stress proteins	15
1.1.5. Accumulation of solutes	15
Potassium (K ⁺) ions	16
Amino acids	18
Sugars	21
Polyols	22
Quaternary ammonium compounds	25
1.2. Responses to low-temperature stress	31
1.3. Responses to high-temperature stress	34
1.4. Aim of the present study	35

Chapter 2	Molecular cloning and characterization of the <i>codA</i> gene for choline oxidase from soil bacterium <i>Arthrobacter globiformis</i>	37
2.1.	Summary	38
2.2.	Introduction	39
2.3.	Materials and Methods	40
2.4.	Results and discussion	45
Chapter 3	Expression of the <i>codA</i> gene in a cyanobacterium <i>Synechococcus</i> sp. PCC 7942	51
3.1.	Summary	52
3.2.	Introduction	53
3.3.	Materials and Methods	54
3.4.	Results	59
3.5.	Discussion	65
Chapter 4	Evaluation of the effects of glycinebetaine in enhancing the tolerance to salt and temperature stresses	67
4.1.	Summary	68
4.2.	Introduction	69
4.3.	Materials and Methods	70
4.4.	Results	72
4.5.	Discussion	93
References		98

Acknowledgments

The author expresses her profound gratitude to Professor Norio Murata, Division of Cellular Regulation, National Institute for Basic Biology; Department of Molecular Biomechanics, The Graduate University for Advanced Studies, for valuable discussions, support and encouragement throughout the work and for critical reading the manuscript.

The author is very grateful to Drs. Yoshitaka Nishiyama and Dimitry A. Los, Division of Cellular Regulation, National Institute for Basic Biology, Professor Hidenori Hayashi, Department of Chemistry, Ehime University, and Dr. Zoltan Gombos, Biological Research Center of the Hungarian Academy of Science, Szeged, for their guidances and helpful discussions.

The author wishes to thank Dr. Laszlo Mustardy, Biological Research Center of the Hungarian Academy of Science, Szeged, for estimation of the cell volume of *Synechococcus* strains PAM and PAMCOD, Dr. Susan S. Golden, A&M Texas University, for kindly providing plasmid pAM1044, Mr. Shoichi Higashi, National Institute for Basic Biology, for guidance in lipid analysis, and Ms. Yumiko Makino, the Center for Analytical Instruments, National Institute for Basic Biology, for determinations of amino acid sequence and concentration of glycinebetaine.

Abstract

The roles of glycinebetaine in enhancing tolerance of a cyanobacterium to environmental stresses are studied.

The previous studies on responses of organisms against salt and temperature stresses are reviewed in Chapter 1.

Chapter 2 describes the molecular cloning of the *codA* gene for choline oxidase that oxidizes choline to glycinebetaine, from a soil bacterium *Arthrobacter globiformis*.

Transformation of the cyanobacterium, *Synechococcus* sp. PCC 7942 with the *codA* gene is described in Chapter 3.

The characterization of the transformed cells on their tolerance against environmental stresses is described in Chapter 4. The results demonstrated that the transformed cells accumulated glycinebetaine at intracellular level of 80 mM, and acquired the ability to tolerate salt stress and temperature stress as evaluated in terms of growth and photosynthetic activity.

Chapter 1

General Introduction

Responses of the cell to environmental stresses

1.1. Responses to salt stress

Lipid bilayer of the cytoplasmic membranes of organisms functions as a barrier to maintain the optimum concentration of solutes in the cytoplasm. It is permeable to water but not to most of other metabolites. When the cells are exposed to a high concentration of salt such as NaCl, water is instantaneously effluxed from the cells upon the degree of osmotic pressure, and the loss of water is accompanied by the decrease of cytoplasmic volume which is known as plasmolysis. These phenomena are generally called osmotic stress. The salt shock also causes the rapid entry of salt into the cells as is called ionic stress. The loss of intracellular water and the invader of salt result in the inhibition of several biological processes, for instance, nutrient uptake, dissociation of proteins, inhibition of photosynthesis, and replication of DNA (Kirst 1989, Csonka 1989).

However, a number of organisms can acclimate and acquire the ability to grow under these conditions. The mechanism of acclimation against salt stress has been studied in a number of halotolerant bacteria, alga, and plants. The salt sensitivity of the cells can be overcome by two types of processes, the steady-state response which can maintain the constant osmolarity during the growth of the cells, and the transient response which occurs after the

changes in the external osmolarity (Csonka 1989). The responses to salt stress may vary in different species. However, the mechanisms in terms of increase in capacity of ion transports, induction of synthesis of stress-induced proteins, and accumulation of osmolytes, are common in most organisms.

1.1.1. Induction of ion-transport activity

Exposure of the cells to high concentrations of salt, typically NaCl, results in a rapid increase of Na⁺ ions in the cytoplasm, leading to the disturbance of ion homeostasis and consequently inhibition of cell growth. One of the important adaptive responses against the increase of external Na⁺ level is to enhance the capacity of Na⁺ transports. Na⁺-ATPase and Na⁺/H⁺ antiporter have been well known as Na⁺ transporters found in both eukaryote and prokaryote (Fig. 1-1). However, in some bacteria such as *Escherichia coli*, Na⁺-ATPase has not been found so far. Na⁺ transport is essential for the cells to maintain cellular homeostasis, for instance, regulation of cytoplasmic pH, osmoregulation, maintenance of low concentration of Na⁺ in the cells, and generation of electrochemical Na⁺ gradient that drives Na⁺/substrate symport systems (Padan and Schuldiner 1994a and 1994c). The energy for Na⁺-ATPase activity is mediated from the hydrolysis of ATP. This transporter is called primary Na⁺ pump. Na⁺/H⁺ antiporter, so-called secondary Na⁺ pump, is driven by the electrochemical proton gradient that is generated by H⁺-

ATPase, electron transport, and photochemical reaction
(Padan and Schuldiner 1994c).

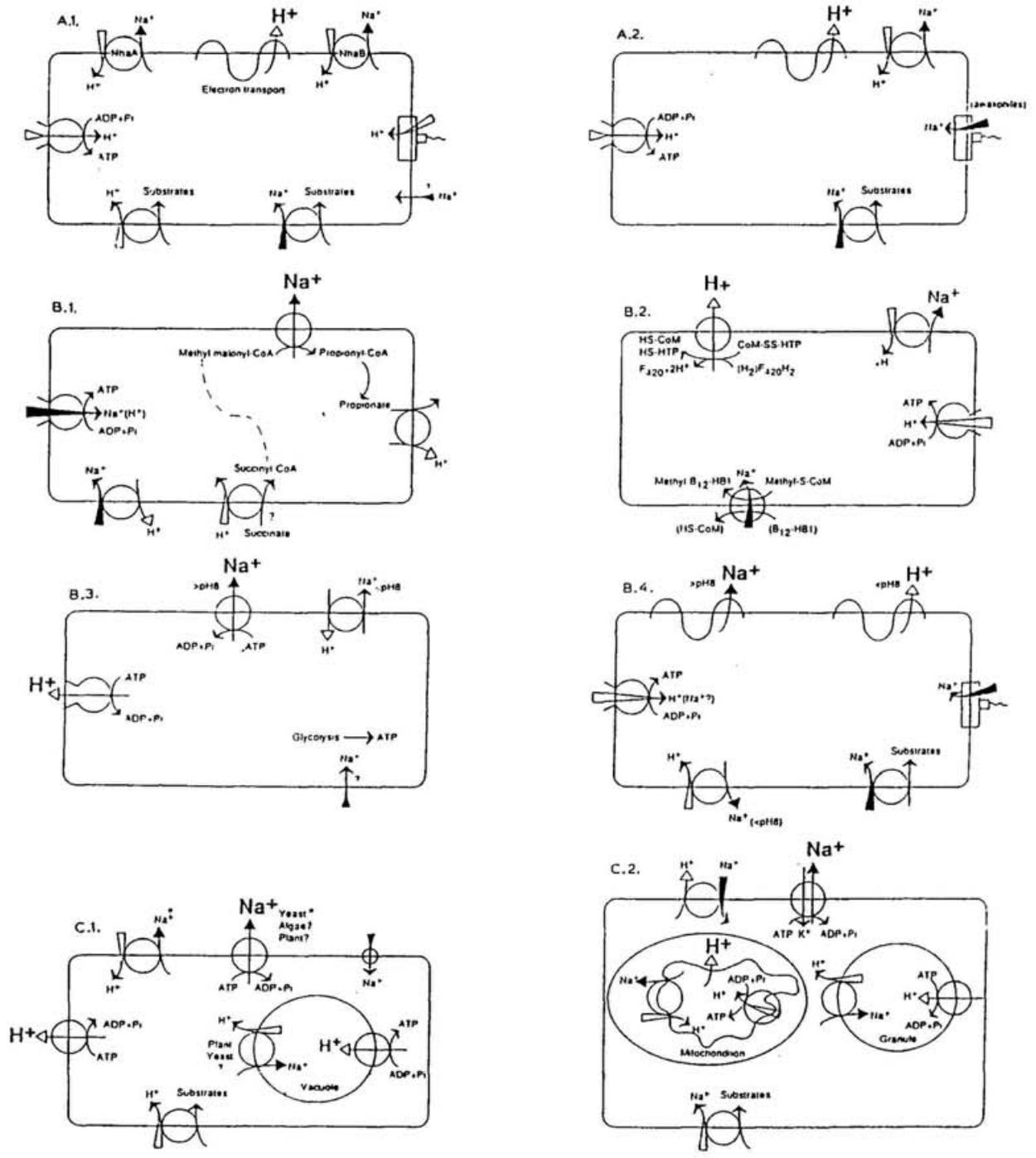


Figure 1-1. The patterns of H^+ and Na^+ cycles in the cells of prokaryotes and eukaryotes. A1 and A2, Bacteria having only Na^+/H^+ antiporter; B1, B2, B3 and B4, bacteria having primary Na^+ pump; C1, higher plants, algae and yeast; C2, animals (Padan and Schuldiner 1994c).

Two genes encoding Na^+/H^+ antiporter, *nhaA* and *nhaB*, have been cloned from *E. coli* (Goldberg et al. 1987, Karpel et al. 1988, Pinner et al. 1992). A hydropathic evaluation of the amino acid sequence of both proteins reveals the presence, respectively, of 11 and 12 putative transmembrane-spanning segments linked by hydrophilic segments of variable length.

The regulation of *nhaA* and *nhaB* genes in terms of the adaptive responses to salt stress has been studied by using *E. coli* deletion mutants, Δ *nhaA* and Δ *nhaB*. Only the Δ *nhaA* mutant showed the sensitivity to NaCl, whereas the Δ *nhaB* mutant had the same phenotype as the wild type (Pinner et al. 1993, Pinner et al. 1994). However, the double mutant, lacking *nhaA* and *nhaB*, was more sensitive to NaCl than the Δ *nhaA* mutant. Mackie (1986) showed that the expression of the *nhaA* gene was regulated by the product of *nhaR* gene that was located at downstream of the *nhaA* gene. The mutant lacking the *nhaR* gene was not as resistant to high concentration of NaCl as wild type, even though the *nhaA* gene was present in the chromosomes. The expression of *nhaR* is dependent on the concentration of Na^+ and Li^+ , and alkaline pH. As analyzed by gel retardation, the promoter region of the *nhaA* gene was bound to the NhaR protein that had been partially purified from *E. coli* (Rahav-Manor et al. 1992). The replacement of Glu 134 to Ala in the NhaR protein increased the affinity for Na^+ on nhaR-mediated *nhaA* transcription and translation, suggesting that Glu134 of NhaR protein may be a part of Na^+ sensor (Carmel et al. 1994). However, the interaction between intracellular Na^+

and the expression of *nhaR* gene has not been well understood.

The mutants, Δ *nhaA* and Δ *nhaA* Δ *nhaB*, were sensitive to NaCl. These strains were used to isolate the gene that confers the resistant to NaCl. The *cadA* gene that partially restored Na⁺ resistance in Δ *nhaA* mutant was isolated from alkaliphilic, *Bacillus firmus* OF4 (Ivey et al. 1991 and 1992).

In the yeast *Saccharomyces cerevisiae*, the Na⁺ Li⁺-ATPase is encoded by the *ENA1/PMR24* ATPase gene (Garcia-deblas et al. 1993), and Na⁺/H⁺ antiporter is encoded by *sod2* gene (Jia et al. 1992). Recently the *HAL3* gene was isolated from *S. cerevisiae* by Ferrando et al. (1995). They have shown that the HAL3 protein, which abundantly localizes in the cytoplasm, plays important roles in the regulation of transports of Na⁺, Li⁺ and K⁺ in the yeast cells.

1.1.2. Enhancement of respiration

The enhancement of respiration in response to salt stress has been reported in cyanobacteria, *Anabeana* L-31 (Apte and Thomas 1986), *S. subsalsa* (Gabbay-Azaria et al. 1992), *Synechococcus* sp. PCC 6311 (Fry et al. 1986, Packer et al. 1987, Nitschmann and Packer 1992), and *Synechocystis* sp. PCC 6803 (Jeanjean et al. 1993). Fry et al. (1986) demonstrated that high concentration of extracellular NaCl enhanced the synthesis of cytochrome *c* oxidase, a terminal electron acceptor of respiratory chain, in the cells of *Synechococcus* sp. PCC 6311. The oxidation of cytochrome *c*

oxidase and respiratory electron transport are coupled with the extrusion of Na^+ by Na^+/H^+ antiporter. It has been suggested that the enhancement of respiratory activity is required to generate electrochemical proton gradient that is a driving force for extrusion of Na^+ by Na^+/H^+ antiporter to maintain a low concentration of Na^+ in the cytoplasm (Blumwald et al. 1984, Paschinger 1977, Molitor 1986).

1.1.3. Changes in ultrastructure and membrane lipid composition

Alteration of the ultrastructure during the change in external osmotic pressure has been demonstrated in the cell-wall less algae, *Dunaliella salina*. This algae can grow under a wide range of NaCl concentration from 0.1 to 5.5 M. When the cells of *D. salina* were transferred from the normal growth medium that contained 1.71 M NaCl to the medium that contained 0.86 M NaCl, the expansion of plasma membrane, chloroplast and other organelles were observed within 2-4 min, and the shape of the cells changed from oval to spherical shape. By contrast, when the concentration of NaCl in the medium was increased above 1.71 M, the cell volume was decreased rapidly (Maeda and Thomson 1986). The rapid expansion of plasma membrane and chloroplast and the decrease of cell volume may need the quick supply and removal of proteins and lipids in the membranes. Using the 5-nitroxide stearate and 16-nitroxide stearate as a spin probe, Curtain et al. (1983) observed a movement of lipids into or out from their reservoir when the cells were

subjected to hyperosmolarity or hypoosmolarity. Einspahr *et al.* (1988a and 1988b) found that the content of phospholipid in the cells decreased dramatically under hyperosmolarity. Based upon the studies using electron microscopy, Maeda and Thompson (1986) proposed that the source of membranes for the expansion of plasma membranes was the small vesicles in the cytoplasm, and the source of membrane for the expansion of chloroplast was endoplasmic reticulum. Memon *et al.* (1993) demonstrated that the movement of the small vesicles was controlled by a 28-kDa GTP-binding protein H^+ -ATPase.

In the presence of high concentrations of NaCl, halotolerant and moderately halophilic bacteria increase the proportion of negatively-charge phospholipid that is a major lipid of bacterial membranes, and also increase the proportion of branched-chain fatty acids (α -15:0, α -17:0 and α -19:0) or cyclopropanoid fatty acids that are known to enhance the fluidity of the membranes (Kates 1986, Russell *et al.* 1986). The increase in the proportion of negatively-charge phospholipid results from the increase in the content of cardiolipin, phosphoglycerolipid glycerophosphoryl diglucosyl diacylglycerol, and the decrease in the content of phosphatidylglycerol (Miller 1985, Hanna, *et al.* 1984). Change in the content of those lipids results in the abundance of negative charges on the lipid membranes that are required for neutralizing the high concentration of Na^+ and decreasing the ion permeability of the membranes (Haest *et al.* 1972).

The change in the physical properties of plasma and thylakoid membranes in response to salt stress has been also

observed in the cyanobacterium, *Synechococcus* sp. PCC 6301 (Riviere et al. 1990, Khomutov et al. 1990). Plasma membranes of the cells which had adapted a high-salt concentration contained a higher lipid/protein ratio compared to the membranes from non-adapted cells. Fry et al. (1986) suggested that the increase in lipid content of the plasma membranes enhanced the activity of cytochrome c oxidase that was involved in the extrusion of Na⁺.

Candida membranefaciens is a holotolerant yeast that can grow under as high concentration of NaCl as 1 M. When the cells of *C. membranefaciens* were subjected to 1.35 M of NaCl, the level of linolenic acid (C18:3) significantly increased and the level of palmitoleic (C16:1) decreased. Among phospholipids, the levels of phosphatidylinositol and phosphatidylethanolamine were elevated, while the levels of phosphatidylcholine and cardiolipin did not change (Khaware et al. 1995).

Some plants change their cellular ultrastructure in response to salt stress. Tobacco cells develop tight zones of adhesion between the plasma membrane and the cell wall to enhance the strength of resisting to the change of the protoplast volume. By contrast, non-adapted cells reveal little or no adhesion characteristic (Zhu et al. 1993). The adhesion of the plasma membrane to the cell wall is mediated by vitronectin and fibronectin-like proteins that are known to be involved in the cell-matrix adhesion of animal cells (Alberts et al. 1989).

1.1.4. Induction of salt-stress proteins

Salt stress can alter the patterns of protein synthesis of organisms in three ways; inhibition of some protein synthesis, enhancement of some protein synthesis, and induction of synthesis of the specific proteins known as salt-stress proteins (Apte and Bhagwat 1989, Claes et al. 1990, Blomberg 1995).

Several genes encoding salt-stress proteins were isolated from a bacterium *Bacillus subtilis*, for instance, *clpC* (Kruger et al. 1994) and *lon* (Riethdorf et al. 1994) genes. However, these genes were not only induced by salt, but also by heat and nutrient starvation. Voelker et al. (1995) have shown that the expression of those genes are regulated by σ^B factor (Voelker et al. 1995). However, little is known about the function of the salt-stress proteins.

1.5. Accumulation of solutes

The exposure of the cells to salt stress results in the efflux of water to outside of the cells. Several organisms including animals respond to salt stress by accumulation of a limited kind of solutes to restore the cell turgor close to the value before the stress is given. The solutes that are accumulated at very high concentrations in the cytoplasm without perturbation of any cellular processes and can protect delicate macromolecular structures from chemical and physical structure-randomizing factors are called compatible solutes or osmoprotectant (Csonka 1989, Papageorgiou and

10

Murata 1995, Yancey et al. 1982). The compatible solutes include K^+ ions, nitrogen-containing compounds (proline, others amino acids such as glutamine and glutamate, quaternary ammonium compounds, and polyamines) and hydroxyl compounds (sucrose, polyols, and oligosaccharides) (McCue and Hanson, 1990). The accumulation of the same solutes can be found from bacteria to higher plants and animals.

The details of the predominant solutes are described as follows.

Potassium (K^+) ions

K^+ ions generally exist in the cytoplasm. They can act as one of the major intracellular osmolytes to maintain cell turgor under high concentrations of salt. K^+ ions are usually accumulated together with glutamate (Csonka 1989). The accumulation of these solutes occur very rapidly (within 30 min), and the rate of K^+ efflux is dependent on the magnitude of the increase in the extracellular osmolarity (Meury and Kepes 1982, Storey and Wyn Jones 1979). However, the accumulation of K^+ in the cytoplasm during salt stress is a transient response or primary response. Usually the organic solutes are synthesized during the transient response, and these organic solutes are substitutively accumulated in the cells to permanently maintain the osmotic balance with the new environment. And this mechanism is called a steady-state or long-term response. At this state, K^+ ions are excreted to the medium, and they are retained only at the same level as in the prestressed cells.

Transport systems that are responsible for the influx and efflux of K^+ ions have been extensively studied in *E. coli*. Two of the transport systems have been characterized: the Trk system that has a relatively low affinity for K^+ ($K_m = 1.5$ mM), and the Kdp system that has a high affinity for K^+ ($K_m = 2$ μ M). The expression of the Trk system is constitutive, while the activity of the Trk transporter can be enhanced by salt stress (Meury et al. 1985). By using the *E. coli* mutant defective in the Kdp system, seven genes which belong to the Trk system have been isolated. They are as follows: *trkA*, *trkB*, *trkC*, *trkD*, *trkE*, *trkG*, and *trkH*. (Epstein and Kim 1971). Bakker et al. (1987) have demonstrated that the gene products of *trkB* and *trkC* are involved in K^+ efflux but not in K^+ influx.

The components of the Kdp system are encoded by the *kdpABC* operon (Siebers et al. 1987) that is transcriptionally controlled by KdpD and KdpE proteins (Polarek et al. 1987). Under normal condition or during a steady-state growth, the *kdpABC* operon is expressed at very low level. However, when osmotic stress generated by salt and sorbitol is given to the cells, the expression of the *kdpABC* operon is induced dramatically. Transcriptional regulation of the *kdpABC* operon has long been argued. The KdpD protein, a membrane-located sensory kinase that contacts with both the inner and outer membranes of *E. coli*, has been proposed to be a sensor of the change in cell turgor. This protein transmits the signal to KdpE protein that may act as a transcriptional activator of the *kdpABC* operon. The regulatory signal for KdpD is likely to be a

10

turgor pressure from the change of osmolarity in the medium and the level of extracellular K^+ . The fully expression of the *kdpABC* operon occurred when the concentration of K^+ in the medium was high (Csonka 1989)

The potassium transport system of nonanimal eukaryotic cells has been recently studied in the yeast, *Saccharomyces cerevisiae*. The major system for potassium transport is encoded by *TRK1* and *TRK2* genes (Gaber et al. 1988, Ko et al. 1991). The mutant lacking *TRK1* is unable to concentrate K^+ from the medium containing a low concentration of K^+ , whereas, the mutant lacking *TRK2* is able to uptake K^+ via the *TRK1* transporter. The *TRK1* is responsible for most of the high-affinity K^+ uptake (Ko et al. 1991). The *HAL1* gene, which encodes a soluble protein of 32 kDa located in the cytoplasm, has been also proposed to be involved in potassium transport in the yeast cells. The expression of this gene is induced by high concentrations of NaCl, KCl and sorbitol. Overexpression of this gene resulted in accumulation of K^+ ions and consequently enhanced the salt tolerance of the yeast cells. (Gaxiola, et al. 1992).

Amino acids

The accumulation of amino acids has been observed in the cells in a number of organisms when they are grown under high external osmolarity. The accumulation of glutamine, glutamate, γ -aminobutyric acid and proline has been found. Among these amino acids, proline is known as the most effective osmoprotectant.

The accumulation of proline is widely found in bacteria, alga and higher plants (Csonka and Hanson 1991, Delauney and Verma 1993). Proline acts as an osmoprotectant to protect the macromolecules against denaturation (Schobert and Tschesche 1978), and also acts as a scavenger of hydroxyl radicals (Smirnoff and Cumbes 1989).

In bacteria, proline is accumulated by either *de novo* synthesis or uptake from the medium in response to salt stress. Biosynthesis of proline is well characterized in *E. coli*. First, glutamate is phosphorylated to γ -glutamyl phosphate by γ -glutamyl kinase. Then γ -glutamyl phosphate is reduced to glutamic- γ -semialdehyde by glutamic- γ -semialdehyde dehydrogenase. Glutamic- γ -semialdehyde is spontaneously cyclized to Δ^1 -pyrroline-5-carboxylate that is further reduced to proline by Δ^1 -pyrroline-5-carboxylate reductase. The genes for γ -glutamyl kinase, glutamic- γ -semialdehyde dehydrogenase and Δ^1 -pyrroline-5-carboxylate reductase, designated as *proA*, *proB* and *proC* genes, respectively, have been cloned from *E. coli* by Deutch *et al.* (1982 and 1984).

Transport systems of proline, PutP, ProP and ProU, have been characterized in *E. coli* and *Salmonella thyphimurium* (Wood 1988). The PutP system is required only for the transport of proline to supply a carbon or nitrogen source to the cells (Ratzkin *et al.* 1978), and its activity is not affected by osmotic stress (Grothe *et al.* 1986). The ProP and ProU systems are responsible for the accumulation of proline at high levels, and their activities are induced by osmotic stress. The ProP and ProU systems are also involved in the transport of glycinebetaine (Csonka 1989). The

regulation of ProP and ProU system will be described in 1.5.5.

In higher plants, proline is synthesized from either glutamate or ornithin. The biosynthesis of proline from glutamate is catalyzed by a bifunctional enzyme, Δ^1 -pyrroline-5-carboxylate synthase that is functionally identical to γ -glutamyl kinase and glutamic- γ -semialdehyde dehydrogenase of *E. coli*. The gene for this enzyme was isolated from *Vigna aconitifolia* (Hu et al. 1992) and *Arabidopsis thaliana* (Verbruggen et al. 1993). Two enzymatic domains of Δ^1 -pyrroline-5-carboxylate synthase reveal are homologous to the ProA and ProB proteins of *E. coli*. The expression of this gene is induced at very high levels when the plants are subjected to salt stress. Kishor et al. (1995) have demonstrated that overexpression of Δ^1 -pyrroline-5-carboxylate synthase results in increase of proline level and enhancement of osmotolerance in transgenic tobacco. The biosynthesis of proline from ornithin proceeds via the δ -transamination of ornithin to Δ^1 -pyrroline-5-carboxylate that is further reduced to proline. The cDNA for ornithin aminotransferase was isolated from *Vigna aconitifolia* (Delauney et al. 1993). Under normal condition proline is synthesized from ornithin, while under salt-stress conditions proline is synthesized from glutamate (Rhodes et al. 1986, Delauney et al. 1993).

Sugars

Some sugars, such as sucrose, glucosylglycerol and trehalose (α -D-glucopyranosyl- α -D-glucopyranoside), are accumulated in the cytoplasm when the cells are exposed to salt stress. Sucrose has been found to be accumulated in the fresh-water cyanobacteria, *Nostoc muscorum* and *Synechococcus* sp. PCC 6311 (Blumwald et al. 1983b). Glucosylglycerol is a major solute that is accumulated in the cells of *Pseudomonas mendocina* SKB70 (Pocard et al. 1994). Trehalose is known as a reserve metabolite, particularly in yeast and fungi. Recently trehalose has been found to be accumulated in response to diverse stresses, salt, dehydration, frost and heat in several bacteria and yeast. Biochemical studies have shown that trehalose stabilizes proteins and membrane lipids. It is likely that trehalose replaces water bound to the membranes and proteins and stabilizes them by hydrogen bond that combines the hydroxyl group of water with the phosphate group of the lipids or the polar groups of the proteins (Strøm and Kaasen 1993).

The biosynthesis of trehalose involves two enzymes, trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase. The first enzyme converts uridine diphosphate-glucose and glucose-6-phosphate to trehalose-6-phosphate that is then hydrolysed to trehalose by the second enzyme (Strøm and Kaasen 1993). The genes for these enzymes are well characterized in *E. coli*. Trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase are encoded by *otsA* and *otsB* genes which are induced by osmotic and

22

during the stationary phase of growth (Giæver *et al.* 1988, Hengge-Aronis *et al.* 1991, Matin 1991). Both genes are located in the same operon and their transcriptions are controlled by σ^S factor. In addition, trehalose-6-phosphate synthase is also activated by potassium glutamate and salts of other monovalent cations (Giæver *et al.* 1988). Overproduction of trehalose in the cells of *E. coli* is not controlled by feedback regulation. The cells prefer to excrete trehalose to outside or degrade it into glucose that can be reutilized as a carbon source (Styrvold and Strøm 1991). The excretion of trehalose is carried out by the periplasmic trehalase encoded by *treA* gene (Hengge-Aronis *et al.* 1991, Repoila and Gutierrez 1991). The *treA* gene is induced by osmotic stress and starvation (Boos *et al.* 1990).

Polyols

The accumulation of polyols in response to salt and dehydration stresses is also widely found in many species including bacteria, marine alga, higher plants, and animals. Polyols, either straight-chain metabolites such as mannitol and sorbitol or cyclic polyols such as *myo*-inositol and its methylated derivatives are suggested to function as osmoprotectants (Bohnert *et al.* 1995).

Biosynthesis of polyols and their roles in protection against stresses have been intensively studied in higher plants. As shown in Figure 1-2, *myo*-inositol is synthesized from glucose-6-phosphate by inositol 1-P synthetase and inositol 1-P phosphatase. Mannitol and sorbitol are

synthesized from fructose 6-phosphate that is converted from glucose 6-phosphate. Myo-inositol 1-phosphate can be phosphorylated to phytate that is utilized as a storage source of phosphate in seed. Inositol 1-phosphate can be utilized for synthesis of phospholipids. Inositol can combine with galactose to form galactinol that serves as a source of galactosyl residues for synthesis of vegetative storage carbohydrates such as stachyose, verbascose and raffinose. Inositol can also be methylated to D-ononitol by inositol O-methyltransferase encoded by *Imt1* gene, and D-ononitol is converted to D-pinitol by ononitol epimerase. These methylated inositols serve as the protective solutes of the plants that are grown under salt and dehydration stresses. The expression of *Imt1* gene is controlled by salt stress and low temperature. In addition, inositol is also involved in the synthesis of cell wall, gums, mucilages and glycoproteins. Phospho-inositides and phosphoinositol are involved in the signal transmission (Bohnert et al. 1995). Methylated inositols have been proposed to serve as the source of methyl group that is used for biosynthesis of quaternary ammonium compounds (Kagan and Clarke 1994).

The accumulation of polyols, sorbitol and inositol, is also observed in animals, mainly in kidney medullary cells which are naturally exposed to high concentrations of NaCl and urea. In animals, sorbitol is synthesized from glucose by aldose reductase. It was shown that the activity of this enzyme was enhanced by intracellular ionic strength (Burg 1994).

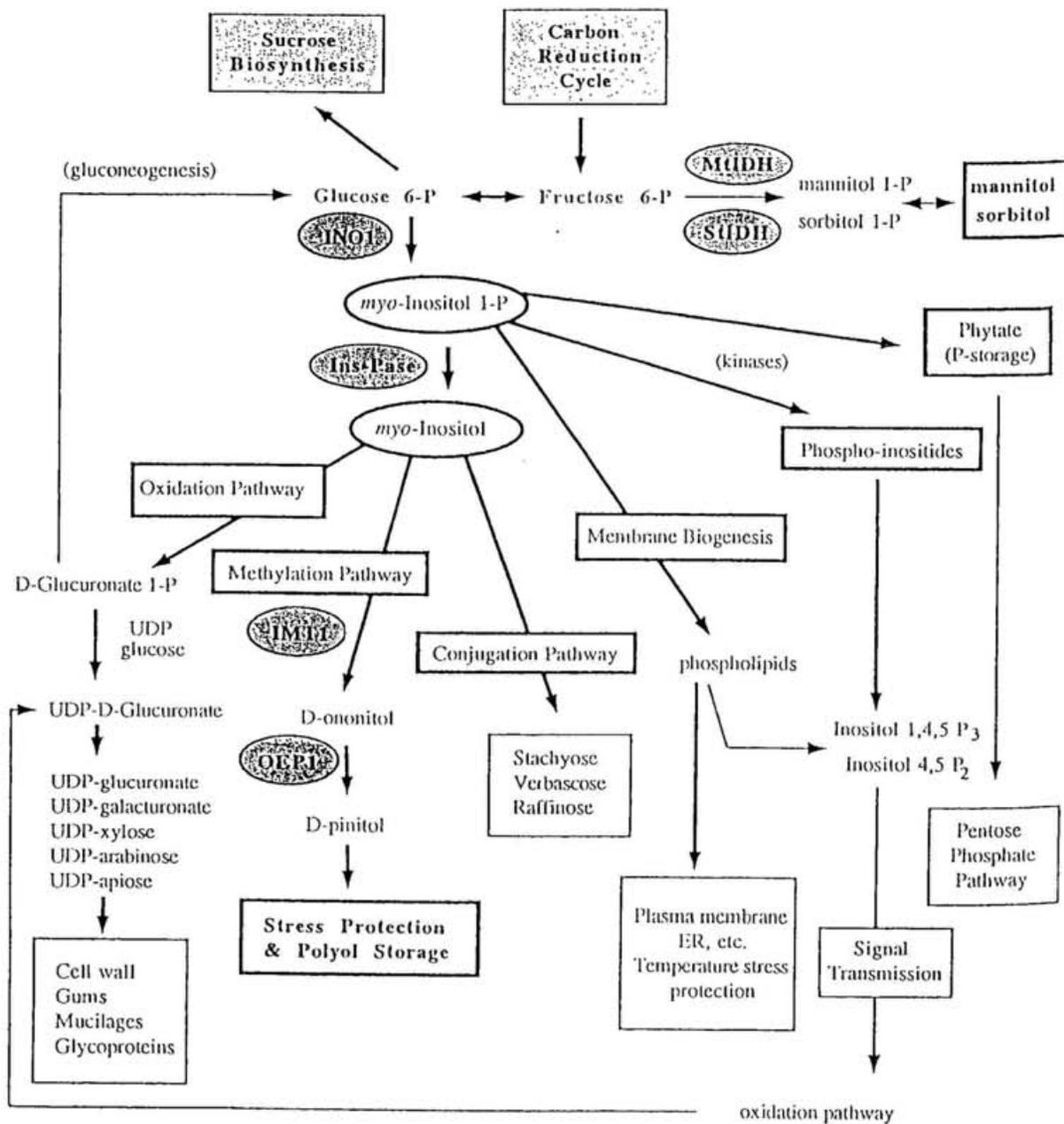


Figure 1-2. Biosynthesis pathway of polyols in higher plants (Bohnert et al. 1995).

Quaternary ammonium compounds

Quaternary ammonium compounds have been recognized to be the powerful osmoprotectants in a variety of organisms. They can be accumulated at high levels in the cytoplasm without perturbation of any biological processes. The structures of these compounds are unique. They contain positive charge and negative charge within a molecule (Table 1-1). Glycinebetaine, choline-O-sulphate, β -alanine betaine, proline betaine and hydroxyproline betaine are known as those compounds (Rhodes and Hanson 1993). Glycinebetaine is found to be the most widespread compound that occurs in a number of organisms including bacteria (Csonka 1989, Gabbay-Azaria et al. 1988), algae (Kirst 1989), higher plants (Rhodes and Hanson 1993), and animals (Burg 1994). The biosynthesis and physiological function of glycinebetaine have been well studied in both prokaryotes and eukaryotes. The involvement of other quaternary ammonium compounds in responses to salt stress has been recently reported (Hanson et al. 1994) in various plants of the Plumbaginaceae family including the species which can adapt to a wide range of salty environments. The structures and biosynthesis pathways of these quaternary ammonium compounds are summarized in Table 1-3.

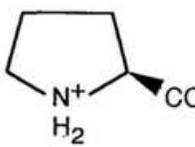
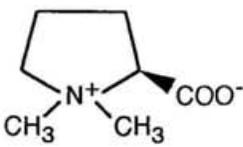
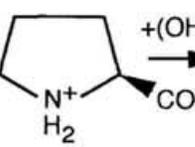
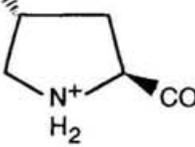
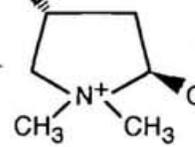
Biosynthetic pathway			Enzyme
$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OH}$	$\xrightarrow{-2\text{H}}$	$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CHO}$	Choline monooxygenase/ Choline dehydrogenase Betainealdehyde dehydrogenase
Choline		Betainealdehyde	
	$\xrightarrow{-2\text{H}}$	$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{COO}^-$	Glycinebetaine
$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OH}$	$\xrightarrow{-4\text{H}}$	$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{COO}^-$	Choline oxidase
Choline		Glycinebetaine	
$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OH}$	$\xrightarrow{+(\text{SO}_3^-)}$	$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{OSO}_3^-$	Choline sulfotransferase
Choline		Choline-O-sulphate	
$\text{H}_3\text{N}^+\text{CH}_2\text{CH}_2\text{COO}^-$	$\xrightarrow{+3(\text{CH}_3)}$	$(\text{CH}_3)_3\text{N}^+\text{CH}_2\text{CH}_2\text{COO}^-$	<i>N</i> -Methyltransferase(s)
β -Alanine		β -Alanine betaine	
	$\xrightarrow{+2(\text{CH}_3)}$		<i>N</i> -Methyltransferase(s)
Proline		Proline betaine	
	$\xrightarrow{+(\text{OH})}$		Prolyl hydroxylase <i>N</i> -Methyltransferase(s)
Proline		Hydroxyproline	
	$\xrightarrow{+2(\text{CH}_3)}$		Hydroxyproline betaine

Table 1-1. The structures and biosynthesis pathways of quaternary ammonium compounds (Rhodes and Hanson 1993, Hanson et al. 1994, Ikuta et al. 1977a)

Glycinebetaine Several bacteria are able to carry out both *de novo* synthesis of glycinebetaine and transport of this compound from the medium by a specific transport system. The biosynthesis of glycinebetaine has been well characterized in *E. coli* and *Arthrobacter globiformis*. In *E. coli*, glycinebetaine is synthesized from choline via the intermediate betainealdehyde. This reaction is catalyzed by two dehydrogenases, a membrane-bound oxygen-dependent choline dehydrogenase and a soluble NAD-dependent betainealdehyde dehydrogenase (Falkenberg and Strøm 1990). The genes encoding these enzymes have been cloned and named *betA* and *betB*, respectively (Andresen et al. 1988, Lamark et al. 1991). The expression of *betA* and *betB* genes is induced by salt stress (Landfald and Strøm 1986). In *A. globiformis*, the oxidation of choline to glycinebetaine is catalyzed by a single enzyme, namely choline oxidase (Ikuta et al. 1977a).

Most of the bacteria are able to transport glycinebetaine from the medium. The active transporters of glycinebetaine, named ProP and ProU, are well characterized in *E. coli* and *Salmonella thyphimurium* (Csonka 1989). These transporters are not only responsible for the uptake of glycinebetaine, but are also involved in the uptake of proline (Csonka 1989) and proline betaine (Haardt et al. 1995). However, it was shown that ProU system was more predominant than ProP system for the uptake of glycinebetaine (Cairney et al. 1985). The proU transporter is encoded by *proU* operon that is composed of three structural genes, *proV*, *proW* and *proX*. The product of the *proX* gene is located in a periplasm. *In vitro* experiments

showed that the ProX protein was able to bind to glycinebetaine. The deduced amino acid sequences of the products of *proV* and *proW* genes show the similarities to the component of the large family of traffic ATPase transporters (Dattananda et al. 1989, Gowrishankar 1989, Gowrishankar et al. 1986). Transcription of *proU* is induced several folds by high osmolarity (Ramirez et al. 1989). In *E. coli* the two promoters, P1 and P2, of the *proV* gene are shown to be responsible for the gene expression in response to osmotic stress. The transcription is regulated by three *trans*-acting factors: (i) P1R that interacts with the P1 promoter, (ii) P2R that interacts with P2 promoter that is initiated by σ^{70} from the RNA polymerase holoenzyme, and (iii) negative regulatory element (NRE) that is required for the repression of *proU* expression at low osmolarity (Dattananda et al. 1991). In addition, it was shown that the abundant nucleoid protein HNS was also involved in the repression of *proU* transcription by interacting with the NRE element (Lucht et al. 1994). The expression of *proU* of *S. typhimurium* is similar to that of *E. coli*, whereas the P1 promoter is not present in *S. typhimurium* (Overdier and Csonka 1992). The mechanism of glycinebetaine transport (OpuA system) has been also studied in *Bacillus subtilis*. OpuA system is encoded by the *opuA* operon comprising three structural genes: *opuAA*, *opuAB*, and *opuAC*. The deduced amino acid sequence of the *opuA* operon shows sequence similarity to the *proU* operon of *E. coli*. The expression of the *opuA* operon is induced by high osmolarity (Kemf and Bremer 1995).

Among higher plants, the biosynthesis of glycinebetaine has been well characterized in sugar beet (*Beta vulgaris*) and spinach (*Spinacia oleracea*). The enzymes involved in the pathway are a ferredoxin-dependent choline monooxygenase that oxidizes choline to betainealdehyde, and NAD-dependent betainealdehyde dehydrogenase that oxidizes betainealdehyde to glycinebetaine (Rhodes and Hanson 1993, Weretilnyk et al. 1989). Both enzymes are predominantly localized in chloroplast (Brouquisse et al. 1989, Hanson et al. 1985, Weigel et al. 1986). However, the minor isoform of betainealdehyde dehydrogenase may exist in the cytoplasm. A cDNA for betainealdehyde dehydrogenase has been cloned from sugar beet, spinach (McCue and Hanson, 1992, Weretilnyk and Hanson 1990), and barley (Ishitani et al. 1995).

In animals, glycinebetaine is generally involved in the catabolism of choline to glycine. The accumulation of glycinebetaine is found in the renal medullary cells of kidney that is naturally exposed to high concentrations of NaCl and urea (Garcia-Perez and Burg 1991). The accumulated glycinebetaine is consequently excreted to urine. Glycinebetaine contained in urine is utilized by bacteria as an osmoprotectant (Kunin et al. 1992). Grossman and Hebert (1989) have demonstrated that choline dehydrogenase, which is usually found in liver and mitochondria, is also present in the renal medullary cells. However, the biosynthesis pathway of glycinebetaine in the renal medullary cells is not well studied. The cDNA (*BGT-1*) encoding glycinebetaine transporter has been cloned from kidney-derived Madin-Darby canine kidney by using *Xenopus* oocyte system (Yamauchi et

al. 1992). *BGT-1* gene encodes a single protein of 614 amino acids. Deduced amino acid of this cDNA shows that the protein is homologous to the transporters of γ -amino-*n*-butyric acid (GABA) and noradrenaline in the brain, suggesting that the *BGT-1* protein is a member of the brain GABA/noradrenaline transporter gene family. The expression of *BGT-1* gene is dependent on Na^+ , Cl^- and also hypotonicity (Uchida *et al.* 1992 and 1993).

Choline-O-sulfate Choline-O-sulfate is synthesized from choline by choline sulfotransferase. This enzyme requires 3'-phosphoadenosine-5'-phosphosulfate as the sulfate donor, and its activity is induced by high concentration of salt. Choline-O-sulfate does not act only as osmoprotectant in Plumbaginaceae, but it also sequesters SO_4^{2-} ions in harmless form. The concentration of SO_4^{2-} ions is usually high in the water source which contains high concentration of NaCl. Members of the Plumbaginaceae contain epidermal salt-secreting glands which can secrete massive amounts of Na^+ and Cl^- ions. However it has a small ability to extrude SO_4^{2-} ions. Thus, the accumulation of choline-O-sulfate would be a mechanism to detoxify SO_4^{2-} ions (Hanson *et al.* 1994).

β -alanine betaine This compound is synthesized via the methylation of β -alanine by N-methyltransferase(s). The accumulation of β -alanine betaine is found in a broader ecological habitat than glycinebetaine, since the

Plumbaginaceae grown in salt marshes accumulates β -alanine betaine but not glycinebetaine (Hanson *et al.* 1994).

Proline betaine and Hydroxyproline betaine These compounds are the derivatives of proline that are effective osmoprotectants accumulated in flowering plants. Proline betaine is synthesized via the methylation of proline by N-methyltransferase(s). The synthesis of hydroxyproline betaine is catalyzed by two types of enzymes, prolyl hydroxylase and N-methyltransferase(s) (Hanson *et al.* 1994).

1.2. Responses to low-temperature stress

The phase transition of membrane lipids has been proposed to be the initial event of chilling injury (Lyons 1973, Raison *et al.* 1973). At normal growth temperature, the plasma membranes and thylakoid membranes form the liquid-crystalline state which is impermeable to ions and small molecules. With decrease in temperature, the membrane lipids get into the phase-separated state and become incapable of maintaining ion gradients, resulting in perturbation of the cellular metabolisms and consequently the death of cells (Murata 1989).

The increase in the fluidity of membrane lipids has been recognized to play an important role in adaptation to low temperature (Murata and Wada 1995). Plants which are sensitive to low temperature contain high levels of saturated molecular species of phosphatidyl-glycerol in their thylakoid membranes, whereas plants which are

resistant to low temperature contain unsaturated fatty acids in almost all the phosphatidylglycerols in the thylakoid membranes (Murata et al. 1982). Similar phenomena have been also observed in cyanobacteria. Sato and Murata (1980 and 1981) have demonstrated that the desaturation of fatty acids is induced by low temperature in *Anabaena variabilis*. A decrease in growth temperature from 38°C to 22°C resulted in decrease of 18:1 and 18:2 fatty acids and increase of α 18:3 in all the major lipid classes. Wada and Murata (1989) showed that in *Synechocystis* PCC6803, a decrease in temperature from 38°C to 22°C increased the degree of unsaturation of C18 fatty acids at the *sn*-1 position of glycerol moiety, but not that of C16 fatty acids at the *sn*-2 position in each lipid class. The desaturation of fatty acids occurs only in the light and is inhibited by chloramphenicol, rifampicin, and 3-(3,4-dichlorophenyl)-1,1-dimethylurea. These suggest that the desaturase activities are induced after a shift from a higher to a lower temperature in cyanobacteria, and that the desaturation of fatty acids is connected with the reactions involved in photosynthetic electron transport.

The effect of the desaturation of fatty acids in the glycerolipids of thylakoid membranes in low-temperature tolerance has been studied by using genetically manipulated *Synechocystis* sp. PCC 6803 (Gombos et al. 1991, Gombos et al. 1992, Wada et al. 1994). Two mutants, Fad 6 and Fad12, lacking the activities of Δ 6 and Δ 12 desaturases which introduce the double bonds to Δ 6 and Δ 12 positions of fatty acids were isolated from *Synechocystis* sp. PCC 6803 (Wada

and Murata 1989, Wada et al. 1990). By using Fad12 mutant, the gene encoding $\Delta 12$ desaturase was cloned from *Synechocystis* sp. PCC 6803, and named *desA*. The Fad6 mutant was transformed with the *desA* gene disrupted with a kanamycin-resistant cassette and the resultant transformant was designated Fad6/*desA*⁻. Among unsaturated fatty acids, Fad6 cells contained mono- and diunsaturated fatty acids and Fad6/*desA*⁻ cells contained only monounsaturated fatty acids, whereas the wild-type cells contained mono-, di and triunsaturated fatty acids. These cells showed no significant difference in growth rate at 34°C. At 22°C, Fad6 cells grew at the same rate as wild-type cells, whereas the growth rate of the Fad6/*desA*⁻ was markedly decreased. In addition, the photosystem II activity of the Fad6/*desA*⁻ cells was more sensitive to photoinhibition at low temperature than that of the wild-type cells (Gombos et al. 1994, Kanervo et al. 1995). These findings suggest that unsaturated fatty acids in membrane lipids play an important role in enhancing the tolerance of cyanobacterial cells to low-temperature stress and photoinhibition at low temperature.

Similar results have been also obtained in the tobacco plants transformed with the gene for glycerol-3-phosphate acyltransferase. This enzyme is involved in the incorporation of fatty acids into glycerol-3-phosphate in higher plants. The transgenic tobacco enhanced the levels of unsaturated fatty acids in phosphatidylglycerol and become more chilling tolerance than the wild type (Murata et al. 1992). These results suggest that the extent of unsaturated

fatty acids in membrane lipids determines the tolerance to low temperature in higher plants as well.

The expression of a number of genes are induced in response to low-temperature stress. These genes are called cold-inducible genes or cold-responsive genes, and are supposed to play an important role in adaptation to low temperature. Those genes have been isolated and characterized from several plant species, such as barley (Catevelli and Bartels 1990, Goddard et al. 1993), wheat (Chauvin et al. 1993, Houde et al. 1992), *Arabidopsis* (Gilmour et al. 1992, Kurkela and Franck 1990, Lang and Palva 1992, Nordin et al. 1993, Orr et al. 1992), *Brassica napus* (Saez-Vasquez et al. 1993, Weretilnyk et al. 1993, White et al. 1994), alfalfa (Wolfraim and Dhindsa 1993, Wolfraim et al. 1993) and cyanobacteria (Sato 1994 and 1995). However, the physiological functions of these proteins have not been well understood.

1.3. Response to high-temperature stress

The molecular mechanism of responses to high temperature has been focused mainly on the induction of a set of heat shock proteins (Hsps). A number of Hsps have been isolated and characterized: Hsp100, Hsp90, Hsp70, Hsp60 and small Hsps. The action of Hsps has been proposed that Hsps interact with denatured proteins and assist in solubilizing them using the energy of ATP hydrolysis for release with refolding of the proteins (Lewis and Pelham 1985). In addition, Hsps are also involved in major growth-

related processes such as cell division, DNA replication, transcription, translation, protein transport, and membrane function (Mager and De Kruijff 1995). The expression of heat shock genes are regulated by σ^{32} , the product of *rpoH* gene that is induced by heat stress (Liberek and Georgopoulos 1993, Bukau 1993).

Plants and cyanobacteria are able to increase the heat stability of photosynthesis in responses to high temperature (Berry and Bjorkman 1980). However, the molecular basis underlying this adaptation is poor understood. Nishiyama *et al.* (1993 and 1994) have suggested that some protein factors present in the thylakoid membranes are involved in this adaptation.

1.4. Aim of the present study

Since glycinebetaine is a preferred osmoprotectant that is accumulated in the cells of many halotolerant plants, algae and bacteria, the physiological function of glycinebetaine has long been investigated. In the cells exposed to salt and dehydration stresses, glycinebetaine can be accumulated at very high levels without perturbation of any biological processes. It has been suggested that the accumulated glycinebetaine can maintain the osmotic balance with the environments and stabilize the organization of proteins and lipid membranes. However, the direct evidence on the role of glycinebetaine in protecting the cells from those stresses has not been demonstrated. It cannot be ruled out that some others chemicals, which are also accumulated

in response to the stresses, play a significant role in the protections.

The present study aims at elucidating the effect of glycinebetaine *in vivo* system. The followings are in the scope of this research. (1) Isolation of the *codA* gene for choline oxidase that converts choline to glycinebetaine, from a soil bacterium *Arthrobacter globiformis*; (2) Transformation of the cyanobacterium *Synechococcus* sp. PCC 7942 with the *codA* gene to establish the biosynthesis of glycinebetaine in this strain; (3) Evaluation of the effect of glycinebetaine in enhancing the tolerance to salt, low- and high-temperature stresses in the transformed cells.

Chapter 2

Molecular cloning and characterization of the *codA* gene for choline oxidase from a soil bacterium *Arthrobacter globiformis*

2.1. Summary

Choline oxidase of *Arthrobacter globiformis* oxidizes choline to glycinebetaine without a requirement of any cofactors. A gene for choline oxidase was isolated from *A. globiformis* as follows: First, the amino-terminal sequence of 21 amino acid residues of the choline oxidase was determined. A DNA fragment corresponding to this amino acid sequence was amplified by PCR. The obtained DNA fragment was used as a probe for screening a genomic DNA library of *A. globiformis* to clone the gene for the choline oxidase. The gene (designated the *codA* gene) contains an open reading frame of 1.641 kb which encodes a polypeptide of 547 amino acid residues. Based on the sequence analysis and comparison with known flavoproteins, the amino-terminal region of deduced amino acid sequence of choline oxidase is identified as a putative FAD-binding site of the protein.

2.2. Introduction

In the soil bacterium *Arthrobacter* sp., choline oxidase is an enzyme that oxidizes choline to glycinebetaine (Fig. 2-1) that is further utilized as a carbon and nitrogen source. This enzyme was first purified from the cells of *A. globiformis* by fractionations with acetone and ammonium sulfate followed by column chromatography with DEAE-cellulose and Sephadex G-200. The enzyme was 100% specific to choline with a K_m value of 1.2 mM (Ikuta et al. 1977b). It was shown by Ohishi and Yagi (1979) that choline oxidase of *A. globiformis* fluoresced as a yellow band on SDS gel in 7% acetic acid and its absorption spectrum revealed a blue shift of the second absorption band that is a characteristic of flavoproteins. Thus, choline oxidase is classified to be one of flavoproteins.

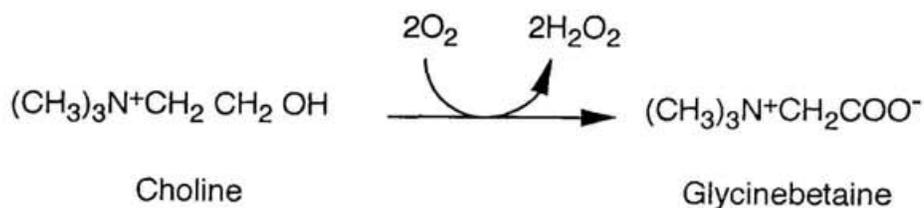


Figure 2-1. Oxidative pathway of choline to betaine by choline oxidase in the cells of *A. globiformis* (Ikuta et al. 1977a).

The gene for choline oxidase was first isolated from *A. pascens*. It was located within a 1.9 kb *BstEII-EcoRI* fragment. Transformation of *E. coli* MC4100, mutant that is defective in glycinebetaine biosynthesis, with 1.9 kb *BstEII-EcoRI* fragment enhanced the osmotolerance of this mutant (Rozwadowski et al. 1991). However, the nucleotide sequence of the gene for choline oxidase from *A. pascens* has not been reported.

In the present work, the gene for choline oxidase (designated *codA* gene) was isolated from *A. globiformis*, and its nucleotide sequence was determined for the first time.

2.3. Materials and methods

Bacterial strain and culture conditions

A. globiformis cells was provided by Asahi Chemical Industry (Shizuoka, Japan). The cells were cultured at 30°C for 20 h in the medium described by Ikuta et al. (1977).

Determination of amino acid composition and partial amino acid sequence

The N-terminal amino acid sequence of choline oxidase (purchased from Sigma Chemical Co., St. Louis, MA, USA) was determined by automatic Edman degradation using a protein sequence analyzer (Model 477A; Applied Biosystems, Foster City, CA, USA). For analysis of internal amino acid sequences, choline oxidase was cleaved into peptide

fragments by cyanogen bromide in 70% formic acid for 14 h (Paul 1990), and the fragments were separated by SDS-PAGE. The peptide fragments were electroblotted onto a polyvinylidene difluoride membrane (AE6660; Atto, Tokyo, Japan) by an electroblotter (Model AE-6675 P/N; Atto, Tokyo, Japan). The membrane was stained with Coomassie brilliant blue, and the peptide fragments was subjected to the determination of the amino acid sequence using the protein sequence analyzer as described above.

Construction of a genomic DNA library

Genomic DNA was isolated from 500 ml of the culture of *A. globiformis*. The harvested cells were once washed with 30 ml of the medium containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl and 1 mM EDTA, and resuspended in 30 ml of the same buffer. Then, they were disrupted by passing through a French press cell (40K; SLM Instruments, Urbana IL, USA) at 160 MPa. The resultant homogenate was treated 5 times with 30 ml of phenol. The aqueous phase was withdrawn and extracted 3 times with the same volume of chloroform to remove the remaining phenol and contaminated lipids. Nucleic acid was precipitated by adding 60 ml of ethanol and the pelleted nucleic acid was dissolved in 1 ml of 10 mM tris-HCl (pH 8.0), 1 mM EDTA, and DNA was purified by equilibrium centrifugation on a gradient of CsCl-ethidium bromide (Sambrook et al. 1989) at 500,000 x g for 18 h.

The obtained DNA was partially digested with a restriction enzyme, *Sau3AI*. The digested DNA fragments were

fractionated by electrophoresis on a 0.8% agarose gel. DNA fragments of 9-25 kbp were recovered from the gel with silica matrix (GENECLEAN II kit; Bio 101, La Jolla, CA, USA). The 9-25 kbp fragments were inserted into the *Bam*HI site of the phage vector λ DASHII according to the manufacturer's protocol (Lambda DASHII/*Bam*HI cloning kit; Stratagene, La Jolla, USA). Packaging the library was performed using a standard protocol (Gigapack II gold packaging kit; Stratagene, La Jolla, USA).

Determination of partial sequence of choline oxidase gene by PCR

Based on the N-terminal amino acid sequence of choline oxidase, 5 amino acids counted from both ends of 21 amino acid sequence were selected for synthesis of oligonucleotides (Fig. 2-2). A partial sequence of choline oxidase gene was amplified by PCR using synthesized oligonucleotides as primers and the genomic DNA of *A. globiformis* as a template. The amplification was performed by 30 cycles of 1 min at 94°C for denaturation and 3.5 min at 32°C for annealing and 2.5 min at 72°C for synthesis. The amplified products were subcloned into the T-A cloning vector (TA Cloning™ kit; Invitrogen, San Diego, CA, USA), and their nucleotide sequences were determined by a DNA sequencer (Model 370A; Applied Biosystems, Foster City, CA, USA).

Screening of genomic DNA library

The genomic DNA library of *A. globiformis* was screened with the 50 bp PCR product (the amplified partial gene of choline oxidase) as a probe according to the standard method (Sambrook et al. 1989). About 3,000 recombinant plaques were transferred to the nylon membranes (Gene Screen Plus; Dupont/NEN Research products, Boston, MA, USA). The membranes were hybridized with the 50 bp PCR product which was labeled with ^{32}P using the random-labelling kit (TaKaRa, Kyoto, Japan). The hybridization was performed in the medium containing 1 M NaCl, 0.1 M sodium citrate, 10% dextran sulfate, 5% sodium dodesyl sulfite, 5% denhardt's solution and 100 mg/l salmon DNA at 50°C for 18 h. After hybridization, the membranes were washed with 300 mM NaCl, 30 mM sodium citrate and 0.1% sodium dodecylsulphate at 42°C for 1 h and at 65°C for 30 min, and then exposed to an X-ray film at -70°C for 20 h (WIF50; Konica, Tokyo, Japan).

Characterization of positive clones

The λ DNAs of positive clones were isolated and digested with the restriction enzymes, *Xba*I and *Xho*I. The resultant fragments were subjected to Southern blot analysis. The DNA fragment which hybridized with the probe was isolated from the positive clones and subcloned into a plasmid pBluescript (SK+) (Stratagene, La Jolla, CA, USA). The insert of 3.6 kbp *Xba*I-*Xho*I fragment in pBluescript was mapped with the restriction enzymes *Bst*EII, *Pst*I, *Sac*I, *Sal*I, and *Sma*I. The

fragments from digestions with *Pst*I and *Sal*I, respectively, were separately subcloned into pBluescript and their nucleotide sequences were determined with the automatic DNA sequencer (Model 370A, Applied Biosystems, Foster City, CA, USA).

Southern blot analysis

For Southern blot analysis, 1 µg of DNA was digested with restriction enzymes. The digested DNA fragments were fractionated on a 0.8% agarose gel and transferred to a nylon membrane. The hybridization and washing of the membrane were performed as described above, and the membrane was exposed to an X-ray film.

2.4. Results and discussion

Partial sequence of choline oxidase gene

The amplified products of PCR on 3% an agarose gel showed the size (about 50 bp) which corresponded to the nucleotide sequence reverse translated from N-terminal of choline oxidase. These amplified products were subcloned into T-A cloning vector. Plasmid which contained the insert was selected for determination of nucleotide sequence. The amino acid sequence deduced from nucleotide sequence of the insert is identical to the N-terminal amino acid sequence of choline oxidase. It suggests that this clone contains a part of choline oxidase gene. This fragment was used as a probe

for Southern blot analysis and screening of genomic DNA library.

**Isolation of the gene for choline oxidase from
*Arthrobacter globiformis***

A genomic DNA library prepared from *A. globiformis* was screened with the insert of the selected plasmid. Four hybridizing phage clones were obtained from about 3,000 recombinants. λ DNAs of the phages were isolated and subjected to Southern blot analysis. All four yielded a *Xba*I-*Xho*I fragment of 3.6 kbp that hybridized with the probe. This fragment was subcloned into pBluescript. Figure 2-3 shows a map of the restriction sites of the insert.

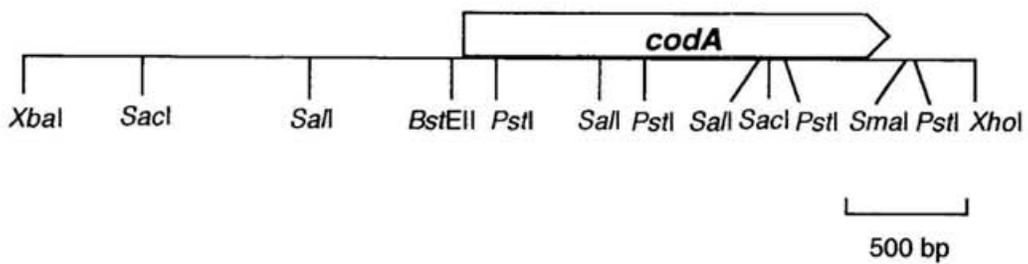


Figure 2-3. The restriction map of the 3.6 kbp *XbaI-XhoI* insert that contained the *codA* gene. The nucleotide sequence was determined in the region from the first *SalI* to *XhoI*.

Nucleotide-sequence analysis

The nucleotide sequence of the region from the first *SalI* to *XhoI* was determined. The nucleotide sequence and deduced amino acid sequence of choline oxidase gene are shown in Figure 2-4. The gene contains an open reading frame of 1.641 kb which encodes a polypeptide of 547 amino acid residues. The deduced amino acid sequences which are identical to the determined N-terminal and internal amino acid sequences of choline oxidase are indicated by underlines. These results suggest that the obtained gene is for choline oxidase of *A. globiformis*. The gene was designated as the *codA* gene.

Since choline oxidase is a flavoprotein (Ohishi and Yaki 1979), we compared its deduced amino acid sequence with those of other flavoproteins, such as methanol oxidase of *Hansenula polymorpha* (Ledebøer et al. 1985), choline dehydrogenase and succinate dehydrogenase of *E. coli* (Lamark et al. 1991, Wood et al. 1984). Figure 2-5 shows that a conserved sequence, GXGXXG, and an array of amino acids typical of an FAD-binding site (Wierenga et al. 1986), both of which are characteristic of flavoproteins, were found in the amino-terminal region of the product of the *codA* gene.

-360 GGGAAATATCCGTCGTCGTAGACGAGCCCTTCGGCCCGTGTAAAGTGGAGACCTTCCACACCGAGGACGAGGCCGTCGCGACCGCCAAAG
-270 ACACCAACTACGGGCTGTCCGGCCCGGTCTGGACCCAGGACCGCCGCAAGACGCAGCCGCGTGGCCGGCCCGCTGGACACGGCACCGTCC
-180 TGGATCAACGACTTCCACCCCTACCTCCACAGACCCAGTGGGGCGCTTGGCCAGTCCGGCGTCGGCCGGCAACTCGGGCCGACCGGC
-90 CTGGCCGAGTACCAGGAGGCCAAGCACATCTACCAGAACACCGCCCGCAGGTCACCGGCTGGTTCGCTGACCAGGCCAAGGAACTAG

1 ATGCACATCGACAACATCGAGAACCTGAGCGACAGGGAGTTCGACTACATCGTCTGGCGGCGGGTCCGCGGGGGCCGCGTCCGCGCC
1 M H I D N I E N L S D R E F D Y I V V G G G S A G A A V A A

91 CGGCTGAGCGAGGATCCCGCAGTGAGCGTGGCGCTGGTGGAGGCCGCGCCGGATGACCGCGCGTGGCCGAGGTGCTGACGCTGGACCG
31 R L S E D F A V S V A L V E A G P D D R G V P E V L Q L D R

181 TGGATGGAGCTGCTGGAATCGGGCTACGACTGGGACTACCCGATCGAGCCGCGAGGAGAACGGCAACTCCTTCATGCGCCATGCCCGTGGC
61 W M E L L E S G Y D W D Y P I E P Q E N G N S F M R H A R A

271 AAGGTCATGGCCGGCTGCTCCAGCCACAACCTCTGCATCGCTTTCGGGCCCGCGCGAGGACCTGGACGAGTGGGAGGCCAAGTACGGC
91 K V H G G C S S H N S C I A F W A P R E D L D E W E A K Y G

361 GCCACCGGCTGGAAACCGCGAGGGCGCTGGCCGCTGTACAGCGGCTGAAACCAACGAGGACCGGGCCCGGACCGCCGCACCACGGG
121 A T G W N A E A A W P L Y K R L E T N E D A G P D A P H H G

451 GACTCCGGCCCGTGCACCTGATGAACGTGCCCGGAGGACCCGACCGCGTCCGGCTCCTGGACGCTGGGAGCAGGCCGCATCCCG
151 D S G P V H L M N V P P K D P T G V A L L D A C E Q A G I P

541 CGCGCGAAGTTCACACCGGACCCACCGTGGTCAACGGCCCAACTTCTCCAGATCAACCGCGCGCGGACCGCCACCGCTCCTCCAGC
181 R A K F N T G T T V V N G A N F F Q I N R R A D G T R S S S

631 TCGGCTCCTACATCCACCCGATCGTTCGAGCAGGAGAACCTCACCTGCTAACCGGCGCTGGCGCCCGCCAGCTGGTGTTCGACCGGGAC
211 S V S Y I H P I V E Q E N F T L L T G L R A R Q L V F D A D

721 AGGCGCTGCACCGGCGTGCACATCGTGGACTCCGCCCTTCGGCCGACCCATCGGCTGACGGCGCGCAATGAAGTCTGCTCCTCCACCGCC
241 R R C T G V D I V D S A F G R T H R L T A R N E V V L S T G

811 GCGATCGATACCGCAAGCTGTGTATGCTCTCCGGAATCGCCCGCCCGCCACCTCGCGCAGCAGGCATCGAGGTCTCTGGTGGACTC
271 A I D T P K L L H L S G I G P A A H L A E H G I E V L G G L

901 CCCCCGCTGGCGGAGCACCTGCAGGACCCCGGAAGGGCTGGTGCAGTTCGAGGCCAAGCAGCCATGGTCGCCGAGTCCACGAGTGC
301 P R R G R A P A G P P G R R G A V R G Q A A H G R R V H A V

991 GTGGGAGATCGGCATCTTCACCCCCACCGAGGACGGCCCTGGACCGCCCGGACCTGATGATGCACTACGGCTCCGTGCCGCTTCGACATGAA
331 V G D R H L H P H R G R P G P P R P D D A L R L R A V R H E

1081 CACCCGCGGACCGGCTACCCACCCAGGAGAACGGGCTTCAGCCCTACCCCGAACGTCACGGCAGCCCGCTCCCGGGCAGCTGTCCGGC
361 H P A A R L P H H G E R A S A S P R T S R T P A P A A L S G

1171 TCGCGACCGCGACTTCCCGGATAAGCCCATGGTCGACCCGCGCTACTTCACCGACCCAGAGGGCCATGACATGCCCGTTCATGGTCGCC
391 C A A A T S A I S P W S T R A T S P T Q K G H D M R V M V A

1261 GGCATCCGCAAGCCCGCGAAATCGCCCGCCAGCCCGCCATGGCGGAATGGACCGCCCGGAGCTCTCCCGCGGCTCGAGGCGCAGACC
421 G I R K A R E I A A Q P A H A E W T G R E L S P G V E A Q T

1351 GACGAGGAGCTGCAGGACTACATCCGCAAGACGCACAACACCGTCTACCCCGCGTGGCCACCGTGCATGGCGCGGTCGAGGACGAG
451 D E E L Q D Y I R K T H N T V Y H P V G T V R H G A V E D E

1441 ATGTCGCCGCTGCACCCGAGCTGCCGGTCAAGGCGTCCCGGCTGCGCGTGGCGAGCCCTCGGTCATGCCCGAGCAGGTGACCGTCC
481 M S P L D P E L R V K G V T G L R V G D A S V M P E H V T V

1531 AACCCCAACATCACCGTTCATGATGATCGCGAGCGCTGCCGCGACTTATCCGCTCCGCCCGCGCGGTGAACAACGACGGCGGACGCC
511 N P N I T V M H I G E R C A D L I R S A R A G E T T T A D A

1621 GAGCTGAGCGCGGCCCTCGCCTAAGCGGGAGCGGCCAGCGCGGGGCTGTCCGGAACCACTGGCGGGCCCGCATGGGGCCGGACACA
541 E L S A A L A *

1711 ATGCCGGTAACTAAGGGTGGGAAAGTCCCTGCTTCCACACCCCGGTTTGTGACCGCCCGGCGGCAACTGGCCCGGCGGCTAAGCCG
1801 AAGTCTTCCGGGGCGGGCCGATCGCTGCGGGCAGTCCGTCGGCCAGCCGCTGCAGCGTCCCGCGGTAATGGCGGTGTAGGCAGGGA
1891 TCGCGTCCGGGTAGATGTACTCGTTGCGGGCGTGGCGCGCTGCCCCACCGCGCCAGGCCGACAGGACCGGGATCCGAGGGCGGAGA
1981 CGAAGTGGCGTCCGTCGCCCGCCACCGAGCGGTTTCCAGCTCCCGCCCTGCTCCA

Figure 2-4. The nucleotide sequence of the gene for choline oxidase of *Arthrobacter globiformis* and its deduced amino acid sequence. The underlines indicate the amino acid sequences which were directly determined by Edman degradation.

COD	MHIDNIENLSDREF	DYIVV	GGGSAG	AAVAARLSE--DPAVSVALVE	A-GPDDR
MOX	MAIPDEF	DIIIV	GGGSTG	CCIAGRLANLDDQNLTVLIE	-GGENN
CDH	MQF	DYIII	GAGSAG	NVLATRLTE--DPNTSVLLLE	AGGPDYR
SDH	MKLPVREF	DAVVI	GAGGAG	IARALQISQ----SQTCALL-	-SKVFPT
		↑↑↑↑↑	↑	↑	↑
			↑	↑	↑
				↑	
					↑↑↑↑↑

Figure 2-5. A comparison of the amino-terminal sequence of choline oxidase with those of various flavoproteins. COD, choline oxidase of *A. globiformis*; MOX, methanol oxidase of *Hansenula polymorpha* (Ledebouer et al. 1985); CDH, choline dehydrogenase of *E. coli* (Lamark et al. 1991); SDH, succinate dehydrogenase of *E. coli* (Wood et al. 1984). Identical amino acids in choline oxidase and other flavoproteins are indicated by asterisks. Arrows indicate the array of amino acids that is typical of FAD-binding site (Wierenga et al. 1986). The highly conserved amino acid sequence and the putative FAD-binding site are enclosed by small and large rectangles, respectively.

Chapter 3

Expression of the *codA* gene in the cyanobacterium
Synechococcus sp. PCC 7942

3.1. Summary

The *codA* gene of *Arthrobacter globiformis* was inserted into the plasmid pAM1044 that contained the *conII* promoter, a spectinomycin-resistance cartridge, and intergenic regions of the chromosomal DNA of *Synechococcus* sp. PCC 7942 that allowed the integration of the insert into the chromosome by homologous recombination. The plasmid pAM1044/*codA* gene was introduced into the cyanobacterium *Synechococcus* sp. PCC 7942, and the resultant transformant strain was designated PAMCOD. The control strain designated PAM was also produced by transforming *Synechococcus* sp. PCC 7942 with the plasmid pAM1044. Analysis by PCR indicated that the inserts were integrated into the chromosomes and all the copies of native chromosomes had been replaced by the recombinant one. As shown by western blot analysis, the *codA* gene was expressed under the control of a *conII* promoter in the cells of *Synechococcus* strain PAMCOD. The accumulation of glycinebetaine in the cells of *Synechococcus* strains PAM and PAMCOD was determined by ^1H NMR spectroscopy. No traces of glycinebetaine were detected in the cells of *Synechococcus* strain PAM. By contrast, the cells of *Synechococcus* strain PAMCOD accumulated betaine at intracellular levels of 60-80 mM.

3.2. Introduction

Since glycinebetaine has been demonstrated to be the powerful osmoprotectant in many species, the genes responsible for biosynthesis of glycinebetaine are of interest with a view to genetically improving the salt tolerance in salt-sensitive organisms. The *betA* and *betB* genes of *E. coli* have been introduced into the cyanobacterium *Synechococcus* sp. PCC 7942. The genes were expressed at high level in the presence of NaCl. The transformed cells accumulated glycinebetaine at the level of 45 mM, and demonstrated the ability to tolerate salt stress (Nomura et al. 1995). The *betB* gene has been also introduced into tobacco *Nicotiana tabacum*. The gene was fused with *ats1a*, a gene encoding for the small subunit of Rubisco of *Arabidopsis thaliana*, to target the gene product to the chloroplast. The results reveal that this gene was expressed in the tobacco cells, and the transgenic tobacco was able to convert intermediate, betainealdehyde, to glycinebetaine (Holmstrom et al. 1994).

By contrast, choline oxidase is a single enzyme that oxidizes choline to glycinebetaine without a requirement of any cofactors. To genetically establish the biosynthesis pathway of glycinebetaine in *in vivo* system, the *codA* gene of *A. globiformis* has been considered to be an alternative way. For this purpose the *codA* gene was introduced into the cyanobacterium, *Synechococcus* sp. PCC 7942. The gene was expressed under control of the *conII* promoter. Transformed cells synthesized glycinebetaine from the exogeneously

supplied choline, and accumulated at the same level either in the presence of NaCl or in the absence.

3.3. Materials and methods

Organisms and culture conditions

Synechococcus sp. PCC 7942 and its transformants were grown at 30°C in BG11 medium (Stanier et al. 1971) under illumination from incandescent lamps at 70 $\mu\text{E m}^{-2} \text{s}^{-1}$ with aeration by air that contained 1% CO₂. For analysis of concentration of glycinebetaine in the cells of transformants, cells were grown in BG11 medium that contained NaCl at various concentrations plus 30 $\mu\text{g ml}^{-1}$ spectinomycin. Choline chloride (Katayama Chemical, Osaka, Japan) was added to the culture medium to a final concentration of 1 mM as the precursor for the synthesis of glycinebetaine.

Construction of plasmid

The plasmid pBluescript that carried the *codA* gene (Fig. 2-3) was digested with *BstEII* and *SmaI*. The *BstEII*-cohesive end was filled in by the Klenow fragment of DNA polymerase (Takara, Tokyo, Japan). The blunt-ended fragment, which contained the coding region of the *codA* gene and a putative ribosome-binding site, was inserted into the *SmaI* site of plasmid pAM1044 (provided by Dr. Susan S. Golden, Texas A&M University). This plasmid carried the *conII*

promoter, a spectinomycin-resistance cartridge, and intergenic regions of the chromosomal DNA of *Synechococcus* sp. PCC 7942 (Fig. 3-1). The orientation of the gene in the construct was confirmed by restriction analysis with *SacI*.

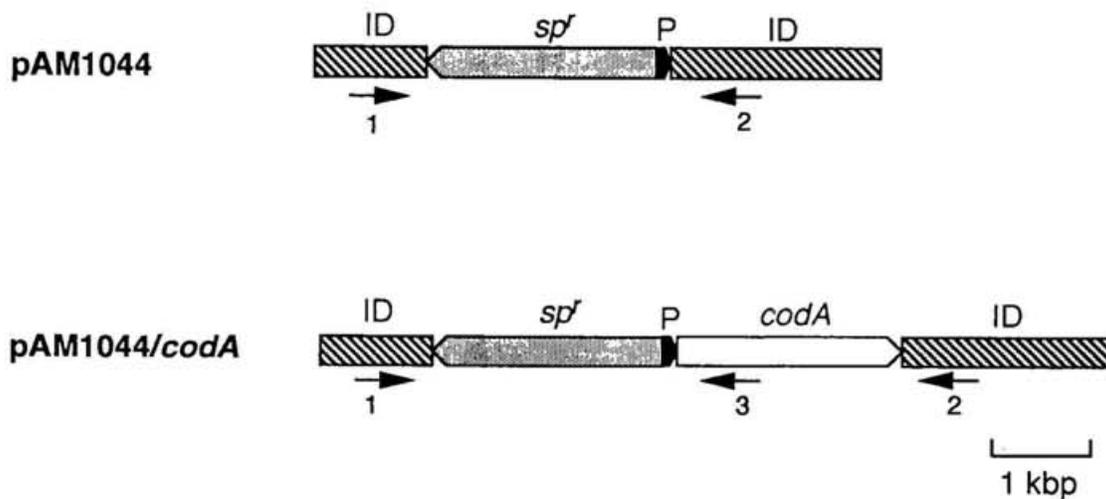


Figure 3-1. Schematic representation of the pAM1044/*codA* construct. ID, Intergenic chromosomal DNA of *Synechococcus* sp. PCC 7942; P, conII promoter; *sp^r*, spectinomycin-resistance gene cartridge. Arrows indicate the primers used for PCR.

Transformation of *Synechococcus* sp. PCC 7942

Cells of *Synechococcus* sp. PCC 7942 were transformed with plasmids pAM1044 and pAM1044/*codA* as described by Elhai *et al.* (1991). Cultures at O.D.730 of 0.33 were harvested by centrifugation at 2,500 x g for 15 min at 25°C. Harvested cells were washed once with 10 ml BG11, and then were resuspended in BG11 to give the cell density of 1.5×10^9 cells/ml. An aliquot of 100 μ l was taken, and 0.1 μ g plasmid DNA was added to the cell suspension. The mixture was incubated at 30°C in the light at $50 \mu\text{E m}^{-2} \text{s}^{-1}$ for 4 h, and then it was spreaded onto the filter membranes (Gene Screen Plus: Du Pont/NEN Research Products, Boston, MA, USA) that were previously placed on agar plates. The plates were incubated at 30°C in the dark for 16 h, and then in the light at $50 \mu\text{E m}^{-2} \text{s}^{-1}$ until the green lawn of the cells appeared. Filter membranes having the green lawn of the cyanobacterial cells were transferred to agar plates that contained 30 $\mu\text{g ml}^{-1}$ spectinomycin in BG11 medium. The single colonies which were resistant to spectinomycin were selected. After several transfers of single colonies to agar plates that contained spectinomycin, the replacement of the native chromosomes by a chromosome that carried the spectinomycin-resistance cartridge and the *codA* gene, was confirmed by PCR.

Western blot analysis

For western blot analysis, total proteins (5 μ g) extracted from the transformed cells, and 25 ng of choline oxidase of *A. globiformis* (Sigma Chemical Co.) were subjected to SDS-PAGE. After electrophoresis, the proteins were electroblotted on a nitrocellulose membrane (BA85; Schleicher & Schuell, Dassel, Germany). Immunological detection was performed according to the protocol supplied with a Vecstain ABC-PO (rabbit IgG) kit (Vector laboratories Inc., Burlingame, CA, USA) using polyclonal antiserum raised in rabbit against the choline oxidase from *A. globiformis* that had been purchased from Sigma Chemical Co.

Quantitation of glycinebetaine

Transformed cells were grown in one liter of BG11 medium supplemented with 1 mM choline chloride in the presence of NaCl at various concentrations plus 30 μ g ml⁻¹ spectinomycin. The cells were harvested and treated with 1 M H₂SO₄ at 25°C for 20 h, and then the cell debris was removed by centrifugation at 3,000 x g for 15 min. The extracted glycinebetaine was recovered from the supernatant by means of the periodide precipitation method (Wall et al. 1960). The resultant glycinebetaine periodide was collected by centrifugation at 3,000 x g and was dissolved in 1 ml of methanol-d₄ (Wako Pure Chemical Industries, Osaka, Japan) that contained 2 mM 2-methyl-2-propanol (Wako Pure Chemical Industries) as an internal standard. This solution was

transferred to an NMR tube, and the ^1H NMR spectrum was recorded at 25°C with an NMR spectrometer (AMX 360 Wb; Bruker, Karlsruhe, Germany) with a pulse time of 5.0 μs and an acquisition time of approximately 4 s. Glycinebetaine was quantified from comparisons of integrated peak intensities (Jones *et al.* 1986). The concentration of glycinebetaine in a cell was calculated from the number of cells in a unit volume and the intracellular volume, which was estimated from electron micrographs of cell morphology.

3.4. Results

Insertion of the *codA* gene into the chromosome of *Synechococcus* sp. PCC 7942

The plasmid pAM1044/*codA* was introduced into *Synechococcus* sp. PCC 7942, and the resultant transformant strain was designated PAMCOD. *Synechococcus* sp. PCC 7942 that had been transformed with pAM1044 alone, designated strain PAM, was also produced for use as a control. Analysis by PCR indicated that all the copies of the native chromosome had been completely replaced by recombinant chromosomes (Fig. 3-2). When the DNA from strain PAM was used as template with primers 1 and 2 (Fig. 3-1), only one band corresponding to a fragment of 2.4 kbp appeared (Fig. 3-2, lane c), suggesting that pAM1044 had been inserted into the chromosomes. The absence of a 400-bp band, which would correspond to the product of PCR from the chromosome of the wild-type strain (Fig. 3-2, lane b), confirmed the complete

replacement of native chromosomes by recombinant chromosomes in strain PAM. When an attempt was made to amplify the DNA from PAMCOD with primers 1 and 2, no bands corresponding to either wild-type chromosomes or recombinant chromosomes were observed. It is likely that the high G-C content of the *codA* sequence impeded amplification by PCR. However, when primers 1 and 3 (Fig. 3-1) were used, a band of 2.6 kbp was amplified (Fig. 3-2, lane d), which corresponded to the calculated size of the amplified product. These findings indicated that the *codA* gene and the spectinomycin-resistance cartridge had been inserted together into the chromosome of strain PAMCOD.

Expression of the *codA* gene in *Synechococcus* strain PAMCOD

The expression of the *codA* gene in strain PAMCOD was examined by western blot analysis (Fig. 3-3). Only one band was detected in the extract from strain PAMCOD that corresponded to a protein with a molecular mass of 60 kDa, namely, the molecular mass of choline oxidase. This result indicated that the *codA* gene had been expressed under the control of the *conII* promoter in *Synechococcus* sp. PCC 7942.

The activity of choline oxidase was also detected in the soluble proteins isolated from cells of *Synechococcus* strain PAMCOD by measurement of the formation of quinoneimine dye, using the peroxidase-phenol-4-aminoantipyrine system as the H_2O_2 acceptor (Ikuta et al. 1977a).

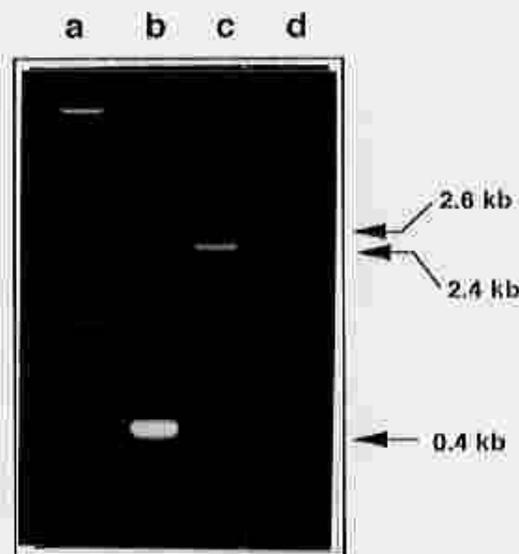


Figure 3-2. Analysis by PCR of the inserted recombinant DNA in the chromosomes of the PAM and PAMCOD strains of *Synechococcus*. Lane a, λ -HindIII/ ϕ x 174-HaeIII fragments (molecular size markers); lane b, the product of PCR obtained with primer 1 and 2 the DNA from wild-type cells of *Synechococcus* sp. PCC 7942 as template; lane c, the product of PCR with primers 1 and 2 and the DNA from cells of strain PAM as template; lane d, the product of PCR with primers 1 and 3 and the DNA from cells of strain PAMCOD as template.

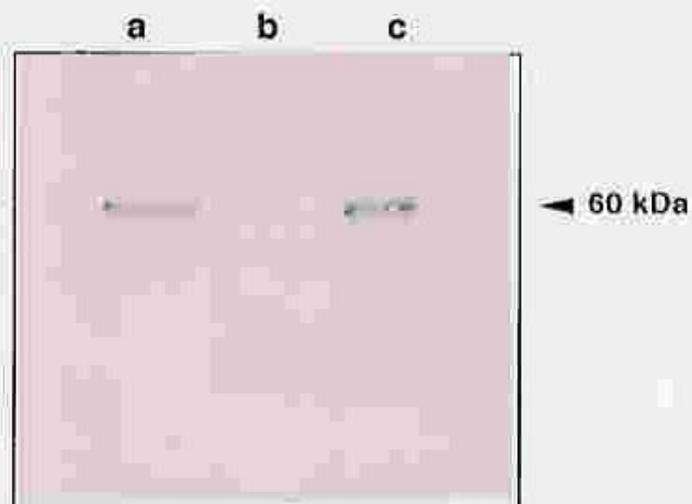


Figure 3-3. Western blot analysis of the expression of choline oxidase in the PAM and PAMCOD strains of *Synechococcus*. Lane a, protein extracted from strain PAMCOD; lane b, protein extracted from strain PAM; lane c, choline oxidase of *Arthrobacter globiformis* purchased from Sigma Chemical Co.

**The accumulation of glycinebetaine in the cells of
Synechococcus strain PAMCOD**

The concentration of glycinebetaine in cells of strains PAM and PAMCOD was determined from the content of glycinebetaine, the number of cells per unit volume and the average volume of the cytoplasm of a cell. The shape of cells, determined by negative staining, was approximated by a cylinder of 0.96 μm in diameter and 2.49 μm in length. The thickness of the cell wall determined by a thin-section method, was 0.07 μm . Therefore, the cytoplasm of a single cell was determined to be equivalent to a cylinder of 0.82 μm in diameter and 2.35 μm in length. The volume of the cytoplasm of a single cell was calculated to be 1.24 μm^3 . Table 3-1 shows the concentrations of glycinebetaine in cells, as estimated in this way. Glycinebetaine was not detected in strain PAM. The concentration of glycinebetaine in cells of strain PAMCOD ranged from 60 to 80 mM at concentrations of NaCl from zero to 0.4 M.

Table 3-1. Concentrations of glycinebetaine in cells of *Synechococcus* strains PAM and PAMCOD. Cells were grown at 30°C in BG11 medium supplemented with 1 mM choline chloride and designated concentrations of NaCl. The results were obtained from two independent experiments.

NaCl	Glycinebetaine	
	PAM	PAMCOD
(M)		(mM)
0	0	60±4
0.2	0	65±6
0.3	0	77±3
0.4	0	77±4

3.5. Discussion

Synechococcus sp. PCC 7942 was transformed with the *codA* gene that was constructed into the plasmid pAM1044. This transformation system allowed the permanent integration of the *codA* gene into the chromosomes of *Synechococcus* sp. PCC 7942. As shown by PCR analysis, the *codA* gene was successfully inserted and replaced all the copies of the native chromosomes of *Synechococcus* sp. PCC 7942. Western blot analysis indicated that the *codA* gene was expressed under the control of the *conII* promoter, which is a constitutive promoter in *E. coli* (Susan S. Golden personal communication). By measurement of choline oxidase activity in the soluble proteins extracted from the cells of strain PAMCOD indicated that choline oxidase was soluble and active in this strain.

Cells of *Synechococcus* strain PAMCOD synthesized and accumulated glycinebetaine up to 80 mM when they were grown in the medium supplemented with 1 mM choline chloride. This result suggests that *Synechococcus* sp. PCC 7942 is able to take up exogeneously supplied choline and that choline is converted inside cells to glycinebetaine by choline oxidase. In several halophilic bacteria, the transport of choline is induced by salt stress, resulting in accumulation of a large amount of glycinebetaine (Boch et al. 1994, Kaenjak et al. 1993). However, we found that the concentration of NaCl in the medium did not significantly affect the level of accumulation of glycinebetaine in cells of strain PAMCOD. It seems likely that the transporter responsible for the uptake

of choline is constitutively operative in *Synechococcus* sp. PCC 7942.

When choline oxidase catalyzes the oxidation of choline to glycinebetaine by molecular oxygen, H_2O_2 is produced simultaneously (Ikuta et al. 1977a and 1977b). H_2O_2 is a hazardous compound in the cell. Scavengers of H_2O_2 , catalase-peroxidase, is present in *Synechococcus* sp. PCC 7942 (Miyake et al. 1991, Shigeoka et al. 1995) and must immediately degrade H_2O_2 when it is synthesized. The details of the regulation of expression of these enzymes by H_2O_2 have not been determined.

Chapter 4

Evaluation of the effects of glycinebetaine in enhancing the tolerance to salt, high- and low-temperature stresses

4.1. Summary

Transformation of *Synechococcus* sp. PCC 7942 with the *codA* gene for choline oxidase established the biosynthesis of glycinebetaine in the cytoplasm of the transformed cells. In this study, the effect of accumulated glycinebetaine in enhancing the tolerance to salt, high- and low-temperature stresses was evaluated. When the cells of *Synechococcus* strains PAM (a control strain) were grown in the presence of 0.4 M NaCl, growth, chlorophyll content and photosynthetic activity of cells of strain PAM were severely retarded. In contrast, cells of strain PAMCOD (a strain having the *codA* gene) could grow under these conditions. Moreover, the PAMCOD cells also could grow at 42°C and 20°C, temperatures at which the growth of the PAM cells was markedly suppressed. Photosynthesis of the PAMCOD cells was more tolerant to low temperature in light and darkness than that of the PAM cells, whereas tolerance of photosynthesis to high temperatures was not affected by the transformation. In parallel with the improvement in the ability of the PAMCOD cells to tolerate low temperature, the lipid phase transition of plasma membranes from the liquid-crystalline state to the gel state shifted toward low temperature. However, the composition of fatty acids of the plasma membranes was not affected by the transformation. The ability of the photosynthetic machinery to recover from low-temperature photoinhibition was enhanced by the transformation.

4.2. Introduction

The physiological functions of glycinebetaine have long been argued. It has been suggested that glycinebetaine protects the cells from salt stress by keeping osmotic balance with the environment (Robinson and Jones 1986), and that glycinebetaine stabilizes the highly organized structure of proteins (Bernard *et al.* 1988, Santoro *et al.* 1992, Winzor *et al.* 1992) for instance it prevents the dissociation of soluble enzymes at high concentration of NaCl (Incharoensakdi *et al.* 1986) and protects the photosystem II complex of the thylakoid membranes against the high salt-induced dissociation of the extrinsic proteins and the manganese cluster (Papageorgiou and Murata 1995). In addition, the accumulation of glycinebetaine was also observed in the cells exposed to chilling stress (Ko *et al.* 1994). However, since glycinebetaine is not the only one compound that is synthesized in the cells under salt and dehydration stresses, a possibility remains that glycinebetaine does not have a direct effect in the protection of the cells against such stresses.

To examine whether the effect of glycinebetaine *in vivo* is direct or not, a system of biosynthesis of glycinebetaine was established in cells of the cyanobacterium, *Synechococcus* sp. PCC 7942, by transformation with the *codA* gene for choline oxidase that oxidizes choline to glycinebetaine (Chapter 3). The resultant transformed cells (*Synechococcus* strain PAMCOD) accumulated glycinebetaine at intracellular

level of 80 mM and rendered the cyanobacterial cells to be tolerant toward salt, high- and low-temperature stresses.

4.3. Materials and methods

Organisms and culture conditions

Synechococcus strains PAM (a control strain) and PAMCOD (a strain having the *codA* gene) were obtained as described previously (Chapter 3). For the evaluation of tolerance to salt stress, cells were grown in BG11 that contained 0.4 M NaCl and 1 mM choline chloride. For the evaluation of tolerance to temperature stress, cells were grown at 30°C or designated temperatures in BG11 that was supplemented with 1 mM choline chloride. The conditions for growth were the same as described in Chapter 3. Growth was monitored in terms of turbidity at 730 nm. Cells at exponential phase of growth at 30°C were used for measurement of photosynthetic activity. The cells at density that corresponded to a chlorophyll concentration of 3-4 $\mu\text{g ml}^{-1}$ were used for light treatments. For measurements of the tolerance of photosynthesis to high and low temperature, the cell density was adjusted to a chlorophyll concentration of 5-10 $\mu\text{g ml}^{-1}$. Chlorophyll concentration was determined by the method of Arnon *et al.* (1974)

Measurement of photosynthetic activity

Photosynthetic oxygen-evolving activity was measured by monitoring the concentration of oxygen at 30°C in BG11 medium that contained CO₂ as an electron acceptor with a Clark-type oxygen electrode (YSI 4004; Yellow Springs Instrument Co., Yellow Springs, Ohio, USA). The activity of photosystem II-mediated electron transport was measured in a similar way in the presence of 1 mM 1,4-benzoquinone as the electron acceptor. Red actinic light at 2,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ was provided from an incandescent lamp after passage through a heat-absorbing optical filter (HA50; Hoya, Tokyo, Japan) and a red optical filter (R-60; Toshiba, Tokyo, Japan).

Analyses of fatty acid and protein composition

Cells of the PAM and PAMCOD strains were grown at 30°C in BG11 medium supplemented with 1 mM choline chloride. Plasma and thylakoid membranes were isolated as described by Omata and Murata (1983). Polar lipids were extracted from plasma and thylakoid membranes by the method of Bligh and Dyer (1959). The fatty acid compositions of membrane lipids were determined as described by Sato and Murata (1988). Protein composition of plasma membranes, thylakoid membranes and a soluble fraction were analyzed by SDS-PAGE.

Assessment of the physical phase of membrane lipids

The lipid phase of plasma membranes in intact cells was monitored by following changes in absorption of carotenoid at 388 nm (Ono and Murata 1981, Wada et al. 1990). Cells were harvested by centrifugation at 3,000 x g for 15 min at 30°C. Pelleted cells were suspended in fresh BG11 medium at a concentration that corresponded to 25 µg Chl ml⁻¹, and then the suspended cells were incubated at designated temperatures for 60 min. Difference-absorption spectra were recorded with a spectrophotometer (Model UV-300; Shimadzu Corporation, Kyoto, Japan), and the increase in absorbance at 388 nm was adopted as a measure of the lipid-phase transition.

4.4. Results

Tolerance of *Synechococcus* strain PAMCOD to salt stress

The ability to tolerate salt stress of the PAM and PAMCOD cells was examined in terms of growth, the accumulation of chlorophyll, and photosynthetic activity.

Figure 4-1 shows the growth of the PAM and PAMCOD cells in the presence of 0.4 M NaCl (concentration corresponds to sea water) and in its absence. Whereas both types of cell grew rapidly, and at about the same rate, in the absence of NaCl, the presence of 0.4 M NaCl remarkably retarded the growth of the PAMCOD cells and completely prevented the growth of the PAM cells. Nonetheless, the results clearly

demonstrated that the PAMCOD cells were much more tolerant than PAM cells to the salt stress.

Figure 4-2 shows the changes in chlorophyll content of the PAM and PAMCOD cells during incubation in 0.4 M NaCl. The chlorophyll content declined slowly in the PAM cells. By contrast, it increased in the PAMCOD cells after a lag period. This result suggests that the PAMCOD cells synthesized and accumulated chlorophyll in 0.4 M NaCl.

Figure 4-3 shows the changes in the photosynthetic activity of the PAM and PAMCOD cells during incubation in 0.4 M NaCl. In both types of cell, the photosynthetic activity decreased during the initial period of incubation in 0.4 M NaCl. However, subsequently, the PAMCOD cells slowly recovered their photosynthetic activity. The results in Figure 4-3 also clearly demonstrate that the PAMCOD cells had acquired tolerance to salt stress.

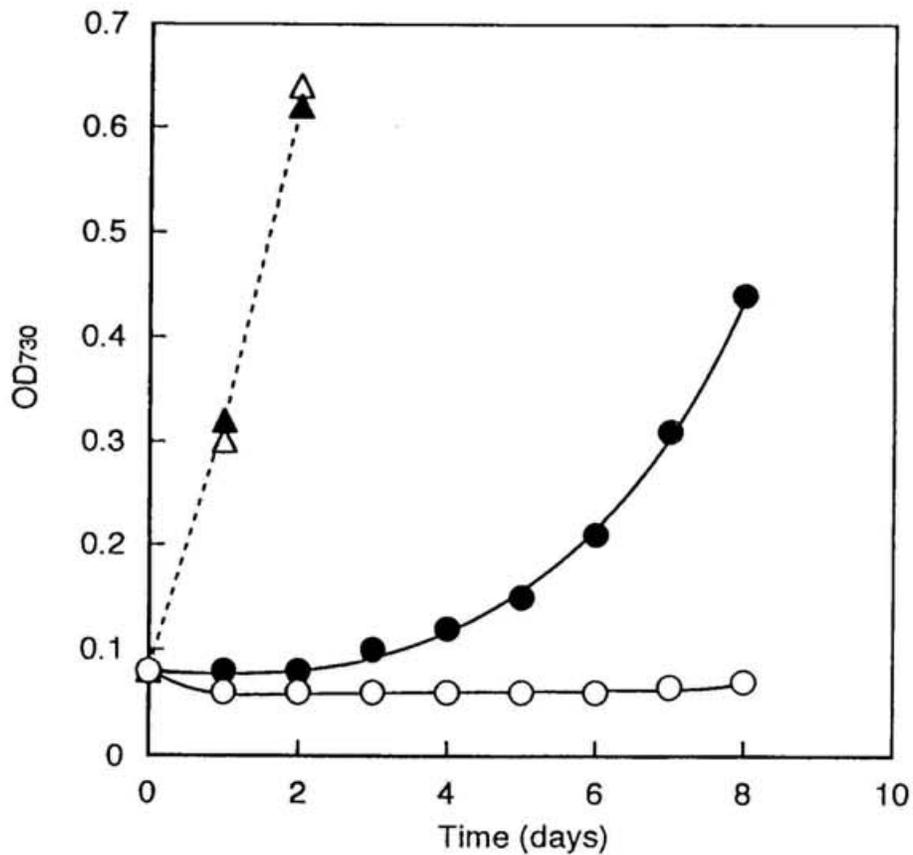


Figure 4-1. Effect of transformation on growth of the PAM and PAMCOD cells in the presence of 0.4 M NaCl. Cells were first cultivated at 30°C for 3 days in the light at $70 \mu\text{E m}^{-2} \text{s}^{-1}$ in BG11 medium supplemented with 1 mM choline chloride. Then the cells were transferred at time zero to BG11 medium supplemented with 0.4 M NaCl and 1 mM choline chloride. In the control experiment, the cells were grown in the absence of NaCl. ○-○, PAM cells in 0.4 M NaCl; ●-●, PAMCOD cells in 0.4 M NaCl; Δ-Δ, PAM cells in the absence of NaCl; ▲-▲, PAMCOD cells in the absence of NaCl.

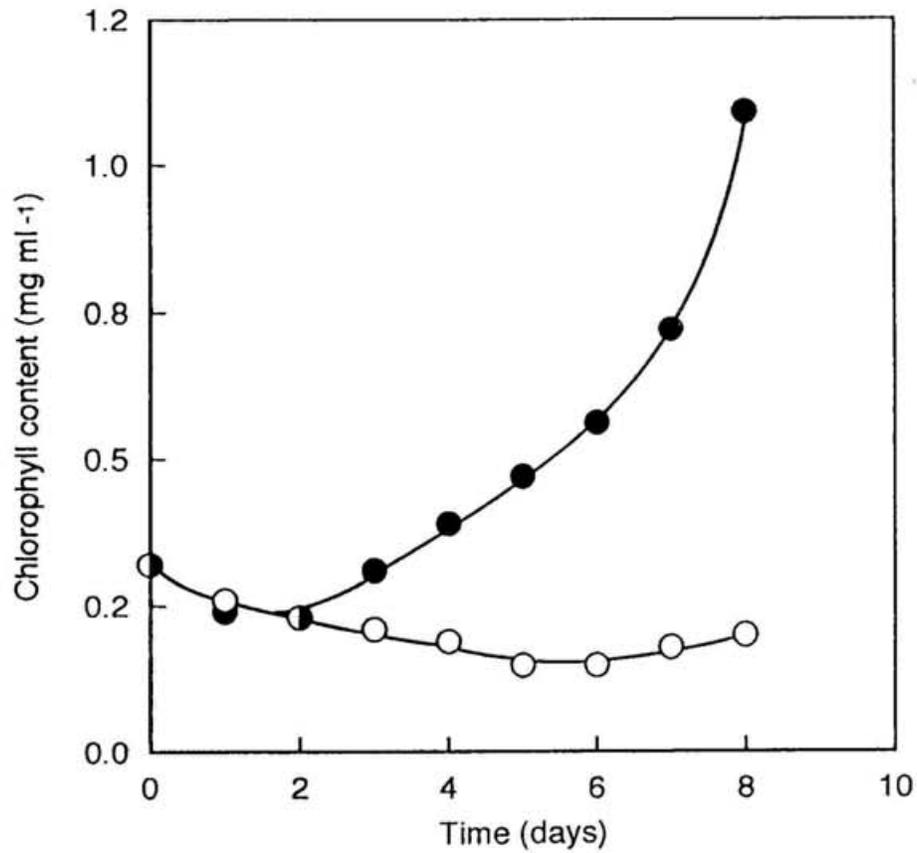


Figure 4-2. Changes in chlorophyll content during growth in the presence of 0.4 M NaCl. Conditions for the initial culture and subsequent growth were the same as those described in the legend to Figure 4-1. O-O, PAM cells; ●-●, PAMCOD cells.

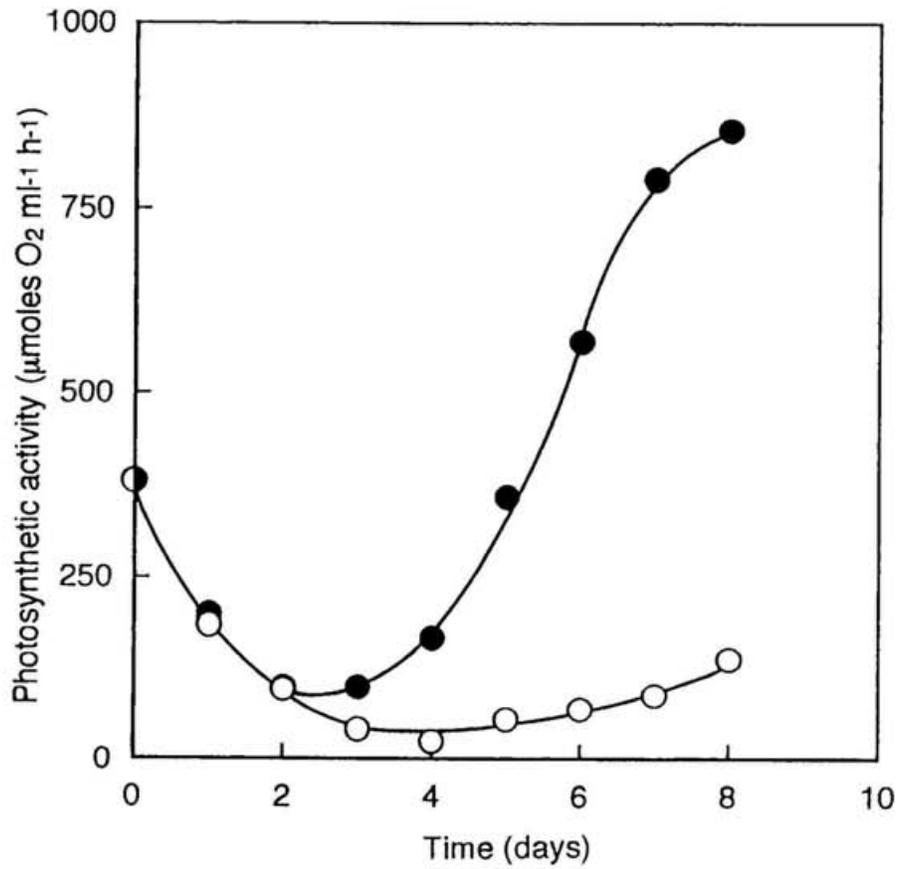


Figure 4-3. Changes in photosynthetic activity during growth in the presence of 0.4 M NaCl. Conditions for the initial culture and subsequent growth were the same as those described in the legend to Figure 4-1. O-O, PAM cells; ●-●, PAMCOD cells.

Tolerance of *Synechococcus* strain PAMCOD to high- and low-temperature stresses

Growth at low and high temperatures

Cells of *Synechococcus* strains PAM and PAMCOD were grown at high and low temperatures in BG11 medium that was supplemented with 1 mM choline chloride. Figure 4-4A shows the growth the PAM and PAMCOD cells at 42°C after transfer from 30°C. The PAMCOD cells stopped growing for two days and then began to grow. By contrast, the PAM cells did not grow at all. When the PAMCOD cells were transferred from 30°C to 20°C, growth stopped for 4 days but then cells began to grow (Fig. 4-4B). PAM cells grew very slowly after a 4-day incubation at 20°C. These results demonstrated that the PAMCOD cells could grow at both higher and lower temperatures than the PAM cells.

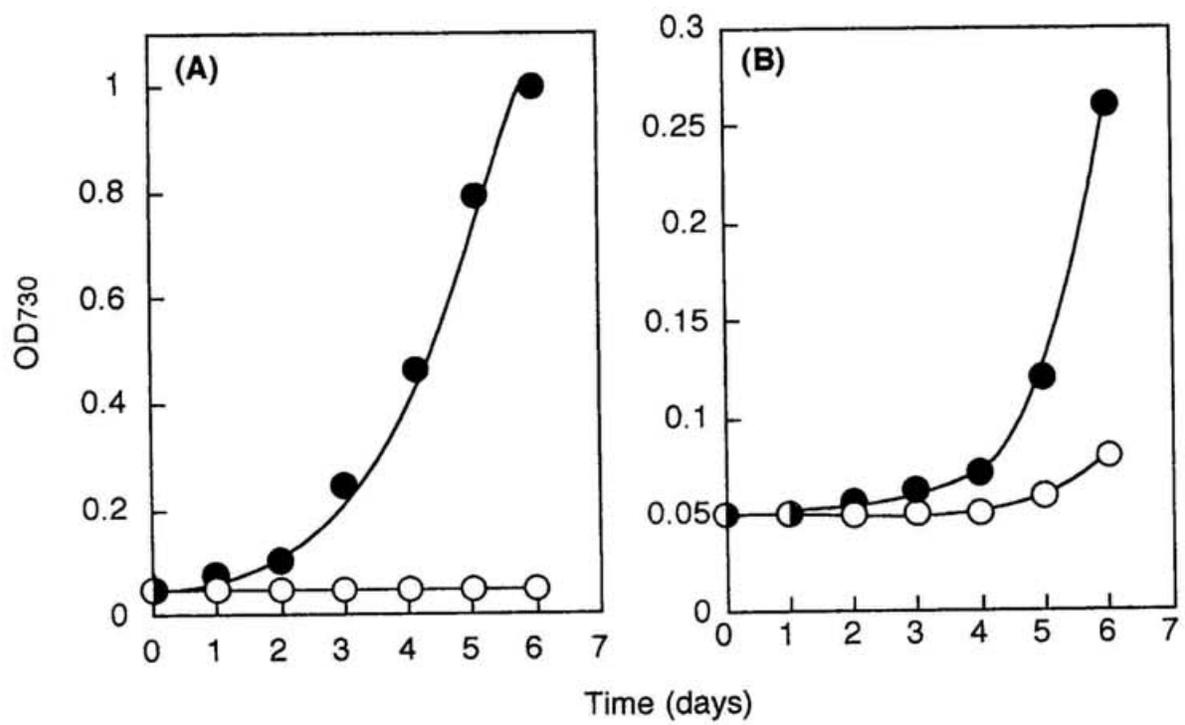


Figure 4-4. Effect of transformation of *Synechococcus* sp. PCC 7942 with the *codA* gene on growth at 42°C (A) and 20°C (B). Growth conditions; light, 70 $\mu\text{E m}^{-2} \text{s}^{-1}$; medium, BG11 supplemented with 1 mM choline chloride. O-O, PAM cells; ●-●, PAMCOD cells.

Inactivation in darkness of photosynthesis at high and low temperatures

The tolerance of the PAM and PAMCOD cells to temperature stress was examined by incubating the cells in darkness at high and low temperatures. Figure 4-5 shows the effect of various high temperatures on the inactivation of net photosynthesis and of the photosystem II-mediated transport of electrons in the PAM and PAMCOD cells. There were no differences in temperature profiles of inactivation between the two types of cells, and the temperature for 50% inactivation was about 50°C in each case.

When the PAM and PAMCOD cells were incubated at low temperature, the photosynthetic oxygen-evolving activity of the PAMCOD cells was more resistant to low temperature than that of the PAM cells (Fig. 4-6A). Similar results were obtained for the photosystem II-mediated transport of electrons. The activity in PAM cells dropped to 50% of the original level at 5°C, while the activity in PAMCOD cells remained at almost the control level at 5°C, starting to decrease only below 5°C (Fig. 4-6B). These results suggested that photosynthesis in the PAMCOD cells was more tolerant to low temperature than in the PAM cells.

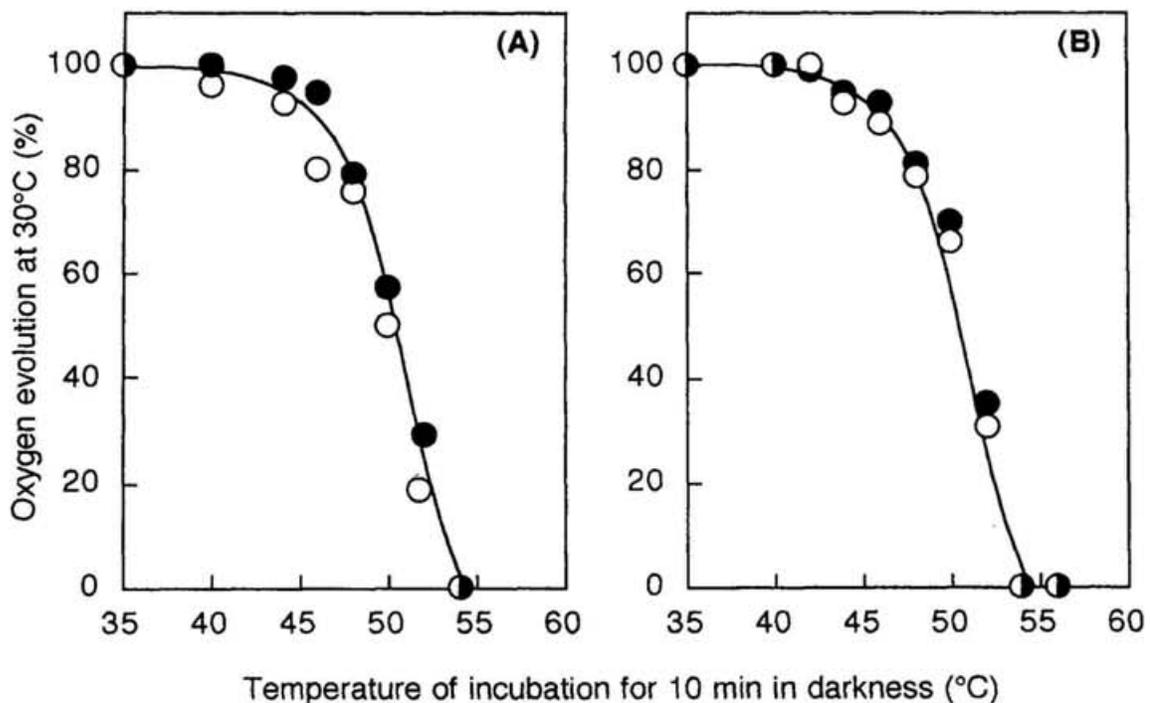


Figure 4-5. Effect of transformation of *Synechococcus* sp. PCC 7942 with the *codA* gene on the high temperature-induced inactivation in darkness of the photosynthetic evolution of oxygen. Cells were incubated at designated temperatures for 10 min in darkness. The photosynthetic oxygen-evolving activity was measured at 30°C in the presence of 1 mM NaHCO₃ (A), and in the presence of 1,4-benzoquinone (B). Absolute values of maximal activities (100%) of the photosynthetic evolution of oxygen in the presence of CO₂ for PAM and PAMCOD cells were 387 ± 23 and 379 ± 19 $\mu\text{moles O}_2$ per mg of chlorophyll per hr, respectively, and those in the presence of 1,4-benzoquinone were 821 ± 46 and 873 ± 28 $\mu\text{moles O}_2$ per mg of chlorophyll per hr, respectively. The values shown are the averages of results from three independent experiments, and the deviation was $\pm 6\%$ at most. O-O, PAM cells; ●-●, PAMCOD cells.

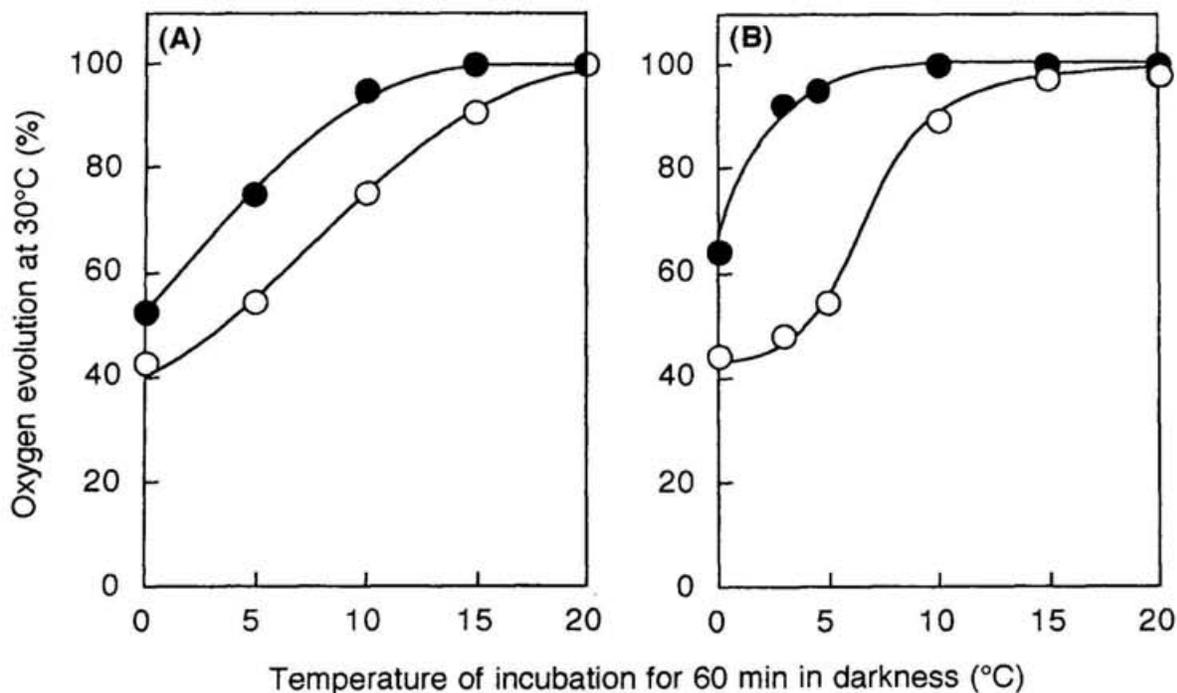


Figure 4-6. Effect of transformation of *Synechococcus* sp. PCC 7942 with the *codA* gene on the low temperature-induced inactivation in darkness of the photosynthetic evolution of oxygen. Cells were incubated at designated temperatures for 60 min in darkness and then incubated at 30°C for 5 min. The photosynthetic oxygen-evolving activity was measured at 30°C in the presence of 1 mM NaHCO₃ (A), and in the presence of 1,4-benzoquinone (B). Absolute values of maximal activities (100%) of photosynthetic evolution of oxygen in the presence of CO₂ for PAM and PAMCOD cells were 387 ± 23 and 379 ± 19 μmoles O₂ per mg of chlorophyll per hr, respectively, and those in the presence of 1,4-benzoquinone were 802 ± 36 and 740 ± 82 μmoles O₂ per mg of chlorophyll per hr, respectively. The values shown are the averages of results from three independent experiments, and the deviation was ± 6% at most. O-O, PAM cells; ●-●, PAMCOD cells.

Inactivation of photosynthesis at high and low temperatures in the light

To examine the effect of light on the inactivation of photosynthesis at high and low temperatures, the PAM and PAMCOD cells were incubated at 42°C and 20°C in darkness or in the light at 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Figure 4-7 shows that there was no difference in the rate of inactivation at 42°C of the photosystem II-mediated transport of electrons between the two strains in the light at either intensity.

During incubation at 20°C, the photosystem II in both types of cell remained fully active in darkness. When the cells were illuminated at 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 120 min, the photosystem II activity of the PAM cells decreased to 35% of the original level (Fig. 4-8A). Although the photosystem II in the PAMCOD cells was also inactivated in the light, the rate of inactivation was much lower than that in the PAM cells (Fig. 4-8A). These results indicated that photosystem II of the PAMCOD cells was more resistant to photoinhibition than that of the PAM cells.

Photoinhibition of photosystem II results from the competition between the light-induced inactivation of the D1 protein and the restoration of the photosystem II complex by incorporation of newly synthesized D1 protein (Aro *et al.* 1990 and 1993). To determine whether the enhancement of tolerance to light stress at low temperature in the PAMCOD cells might be related to suppression of the inactivation or acceleration of the synthesis of the D1 protein,

photoinhibition was induced in the presence of lincomycin that inhibited protein synthesis by interacting with the 50S ribosomal subunit and inhibiting the peptidyltransferase reaction (Fig. 4-8B). In darkness, lincomycin did not affect the photosystem II activity of the PAM and PAMCOD cells. In the light, the inactivation of photosystem II in both types of cell occurred at an identical rate (Fig. 4-8B). This result suggests that the enhancement of tolerance to light stress at low temperature in the PAMCOD cells was not related to suppression of the inactivation of the D1 protein.

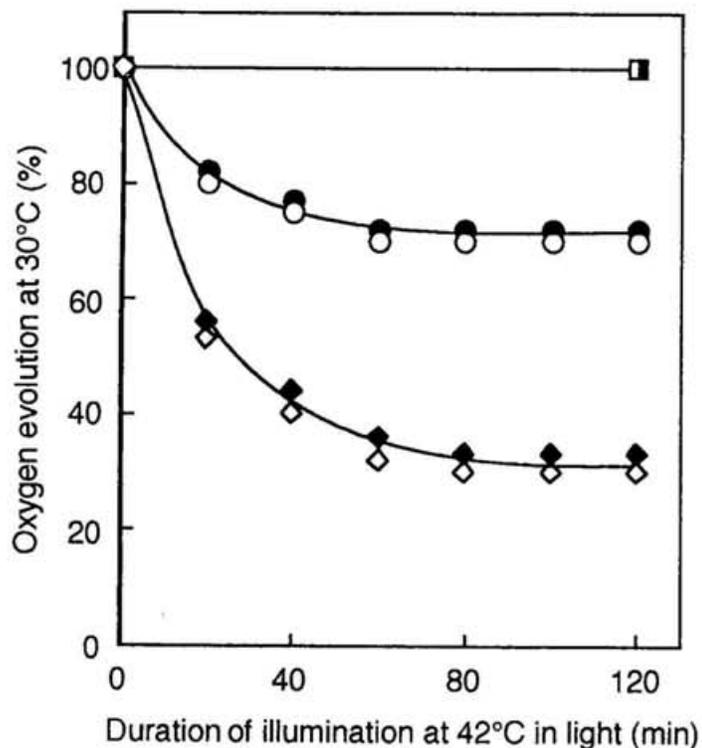


Figure 4-7. Effect of transformation of *Synechococcus* sp. PCC 7942 with the *codA* gene on photoinhibition at 42°C of the photosystem II-mediated transport of electrons. Cells were incubated in darkness or in the light at 500 and 1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Evolution of oxygen due to photosystem II-mediated transport of electrons was measured at 30°C in the presence of 1,4-benzoquinone. Absolute values of maximal activities (100%) of photosystem II for PAM and PAMCOD cells were 698 ± 60 and 688 ± 32 $\mu\text{moles O}_2$ per mg of chlorophyll per hr, respectively. The values shown are the averages of results from three independent experiments, and the deviation was $\pm 5\%$ at most. \square - \square , PAM cells in darkness; \blacksquare - \blacksquare , PAMCOD cells in darkness; \circ - \circ , PAM cells in light at 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$; \bullet - \bullet , PAMCOD cells in light at 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$; \diamond - \diamond , PAM cells in light at 1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$; \blacklozenge - \blacklozenge , PAMCOD in light at 1,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

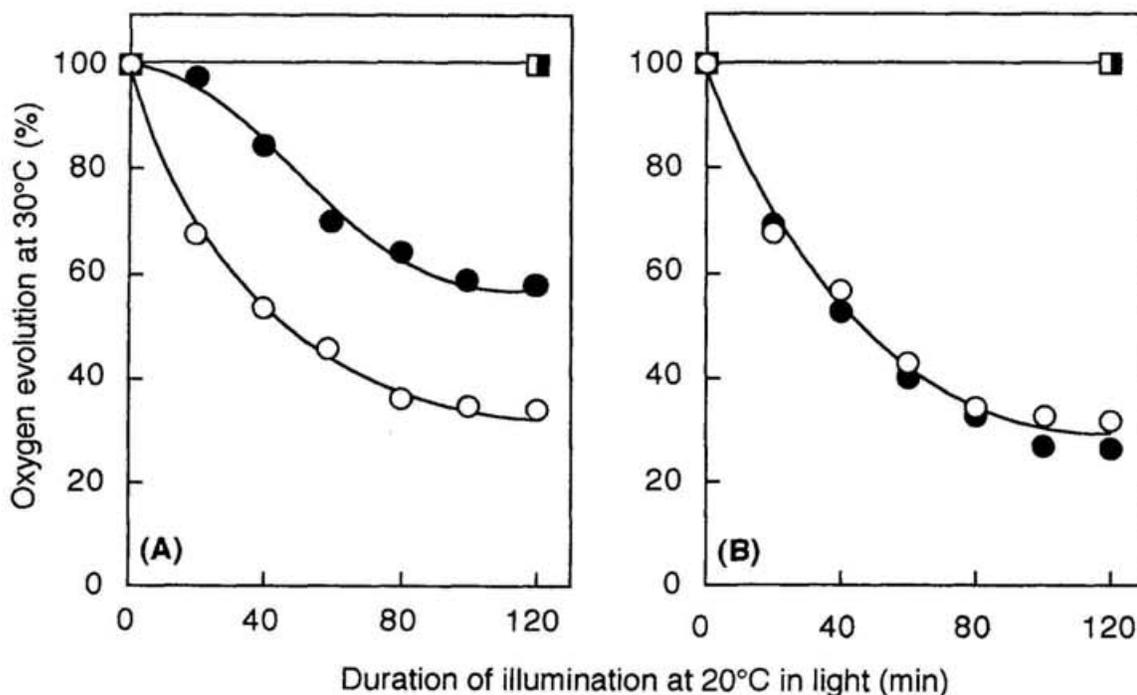


Figure 4-8. Effect of transformation of *Synechococcus* sp. PCC 7942 with the *codA* gene on photoinhibition at 20°C of the activity of photosystem II-mediated transport of electrons. Cells were exposed to light at 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in the absence (A) or in the presence (B) of 400 $\mu\text{g/ml}$ lincomycin. The evolution of oxygen due to photosystem II-mediated transport of electrons was measured in the same way as described in the legend to Figure 4-7. Absolute values of maximal activities (100%) of photosystem II for PAM and PAMCOD cells were 708 ± 60 and 698 ± 32 $\mu\text{moles O}_2$ per mg of chlorophyll per hr, respectively. The values shown are the averages of results from three independent experiments, and the deviation was $\pm 4\%$ at most. \square - \square , PAM cells in darkness; \blacksquare - \blacksquare , PAMCOD cells in darkness; \circ - \circ , PAM in light at 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$; \bullet - \bullet , PAMCOD cells in light at 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Recovery from photoinhibition

The recovery of the photosystem II complex from photoinhibition was evaluated in the PAM and PAMCOD cells. Cells were illuminated with light at $3,500 \mu\text{mol m}^{-2} \text{s}^{-1}$ in order to reduce the photosystem II activity to 15% of the original level. Then the cells were incubated at 20°C or 30°C in the light at $70 \mu\text{mol m}^{-2} \text{s}^{-1}$.

During incubation at 20°C , the recovery of photosystem II activity from photoinhibition occurred only a minimal extend in the PAM cells. By contrast, the photosystem II activity of the PAMCOD cells was restored to 60% of the original level after 120 min (Fig. 4-9A). During incubation at 30°C , the photosystem II activity of both strains were completely restored after 120 min of incubation. However, the rate of recovery in the PAMCOD cells was much higher than that in the PAM cells (Fig. 4-9B).

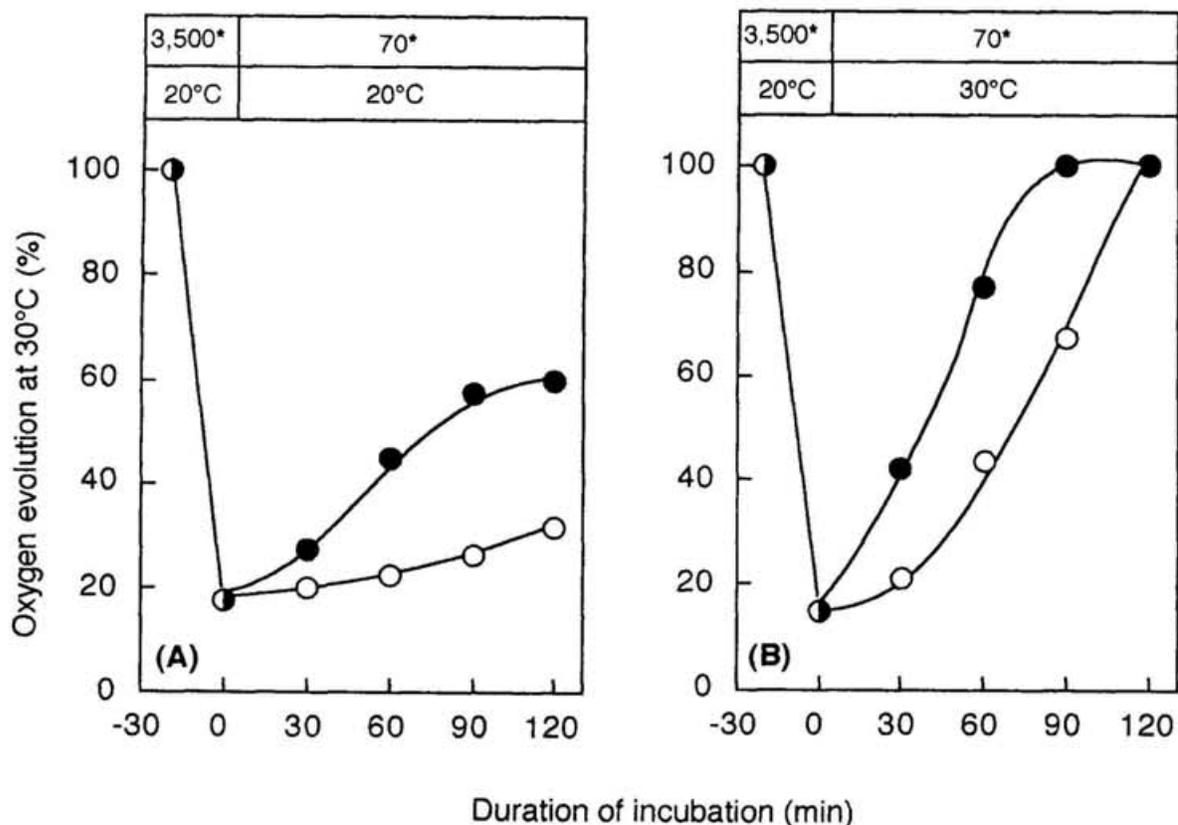


Figure 4-9. Effect of transformation of *Synechococcus* sp. PCC 7942 with the *codA* gene on recovery of the photosystem II-mediated transport of electrons from low-temperature photoinhibition. Cells were first incubated in the light at $3,500 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 20°C for 20 min, and then they were incubated at 20°C (A) or 30°C (B) in the light at $70 \mu\text{mol m}^{-2} \text{s}^{-1}$. The evolution of oxygen due to photosystem II-mediated transport of electrons was measured in the same way as described in the legend to Figure 4-7. Absolute values of maximal activities (100%) of photosystem II for the PAM and PAMCOD cells were 732 ± 78 and $801 \pm 100 \mu\text{moles O}_2$ per mg of chlorophyll per hr, respectively. The values shown are the averages of results from three independent experiments, and the deviation was $\pm 5\%$ at most. O-O, PAM cells; ●-●, PAMCOD cells. *Units are $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Changes in membrane lipids and proteins

The phase transition temperature in the cyanobacterial cells is related to the degree of unsaturation of fatty acids in membrane lipids (Murata 1989). Therefore, the author examined whether the fatty acids in the membrane lipids of PAMCOD cells were more unsaturated than those of PAM cells. Table 4-1 shows the composition of fatty acids in glycerolipids from the plasma and thylakoid membranes of the PAM and PAMCOD cells. There was no significant difference between the two types of cells.

Figure 4-10 shows the electrophoretic patterns of proteins in membrane and soluble fractions that were prepared from the PAM and PAMCOD cells grown at 30°C. With the exception of choline oxidase that was present in the soluble fraction of the PAMCOD cells, the profiles of the soluble proteins from both types of cell were very similar. There were minor differences in the composition of plasma membrane proteins between the PAM and PAMCOD cells. The level of a 14-kDa protein was elevated, and the level of a 16-kDa protein was reduced in the plasma membrane of the PAMCOD cells.

Table 4-1. Effect of transformation of *Synechococcus* sp. PCC 7942 with the *codA* gene on the composition of fatty acids. PAM and PAMCOD cells were grown at 30°C in BG11 medium supplemented with 1 mM choline chloride. The values shown are the averages of results from two independent experiments and the deviation was \pm 3% at most.

	Fatty acid						
	14:0	14:1	16:0	16:1	18:0	18:1(9)	18:1(11)
	(mole%)						
Plasma membranes							
PAM	1	1	54	36	3	2	2
PAMCOD	2	2	53	38	2	2	2
Thylakoid membranes							
PAM	1	2	52	40	2	2	2
PAMCOD	1	2	50	40	2	2	2

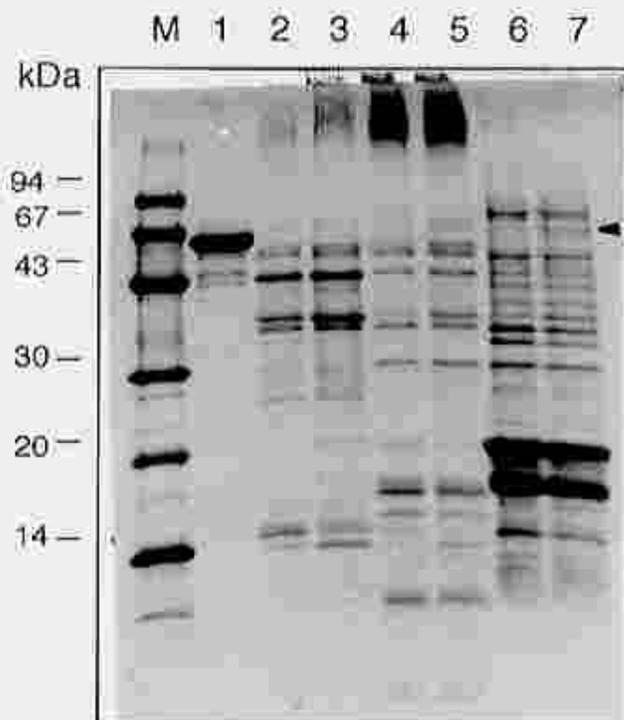


Figure 4-10. Effect of transformation of *Synechococcus* sp. PCC 7942 with the *codA* gene on the composition of proteins in plasma membranes, thylakoid membranes and a soluble fraction. M, Molecular mass markers; 1, choline oxidase (purchased from Sigma Chemical Co., St. Louis, MO, USA); 2, plasma membrane from PAM cells; 3, plasma membrane from PAMCOD cells; 4, thylakoid membrane from PAM cells; 5, thylakoid membrane from PAMCOD cells; 6, soluble fraction from PAM cells; 7, soluble fraction from PAMCOD cells. An arrow indicates the band that corresponds to choline oxidase in the PAMCOD cells.

Phase transition of plasma membrane lipids

The suppression of growth and photosynthetic activity that occurs when the cyanobacterial cells are exposed to low temperature is caused initially by the transition of lipid phase of the plasma membrane from a liquid-crystalline state to a phase-separated state (Murata 1989). Therefore, the author examined whether the enhancement of tolerance to low temperature of the PAMCOD cells was related to the changes in the phase behavior of the membrane lipids.

The transition of lipid phase of plasma membrane of the PAM and PAMCOD cells was examined by monitoring the changes in absorption of carotenoids at 388 nm (Ono and Murata 1981, Wada et al. 1990). Figure 4-11 shows that the phase transition of the membrane lipids of the PAM cells first became apparent at 10°C and ceased at 2°C with a mid-point temperature at about 6°C. By contrast, the phase transition of membrane lipids of the PAMCOD cells began at 5°C with a mid-point temperature at about 3°C. These results demonstrated that the transition of lipid phase of plasma membranes of the PAMCOD cells occurred at a temperature 5°C lower than that of the PAM cells.

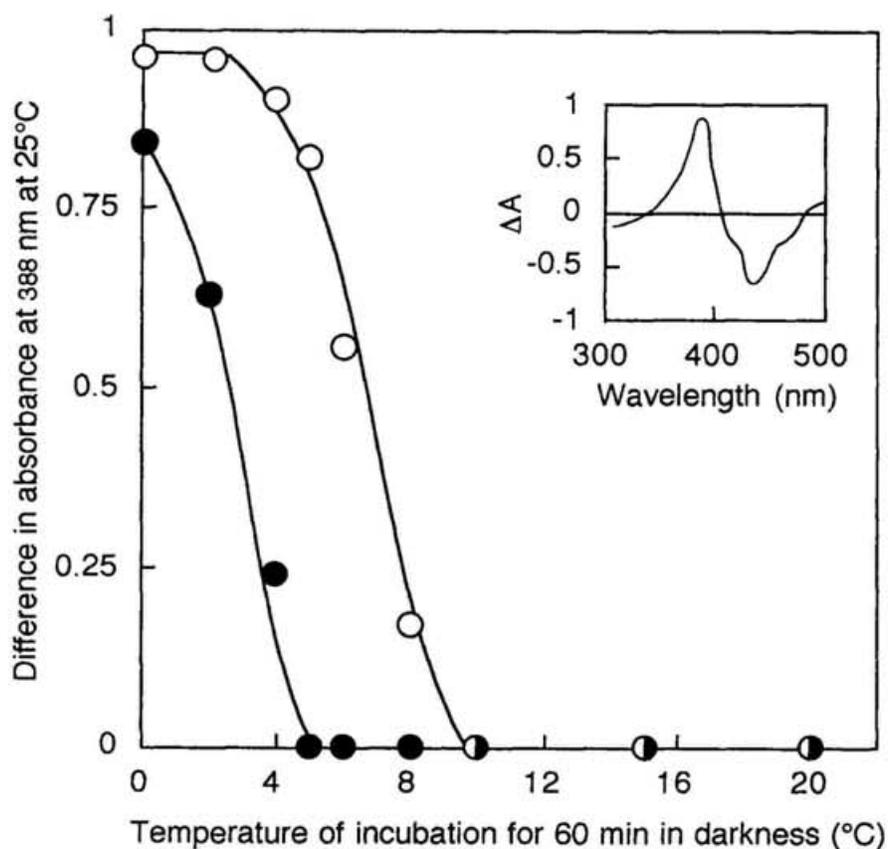


Figure 4-11. Effect of transformation of *Synechococcus* sp. PCC 7942 with the *codA* gene on the low temperature-induced transition of lipid phase of plasma membranes, monitored in terms of changes in absorbance at 388 nm (Ono and Murata, 1981, Wada et al., 1990). Cells at a concentration that corresponded to 25 $\mu\text{g chl ml}^{-1}$ were treated at designated temperature for 60 min in darkness and then the difference-absorption spectra of carotenoids were recorded. O-O, PAM cells; ●-●, PAMCOD cells. Inset, the difference-absorption spectrum at 25°C for PAM cells after incubation at 4°C for 60 min.

4.5. Discussion

Biochemical characteristics of the transformed cells

The cells of the PAMCOD strain that had been transformed with the *codA* gene accumulated intracellular glycinebetaine at about 80 mM, whereas the cells of the PAM strain that had been transformed with the vector alone did not accumulate glycinebetaine (Chapter 3). The biochemical analyses in the present study demonstrated that transformation with the *codA* gene did not affect the fatty-acid composition of membrane glycerolipids (Table 4-1). Moreover, the transformation did not significantly modify the protein composition of cells, although there were minor changes in levels of plasma membrane proteins (Fig. 4-10).

Change in tolerance to salt

As evaluated in terms of growth, chlorophyll accumulation, and photosynthetic activity, the accumulation of glycinebetaine in the PAMCOD cells significantly increased their tolerance to salt stress. The cells of halophilic bacteria accumulated glycinebetaine to maintain an osmotic balance with their environment (Csonka 1989). Higher plants, such as spinach, accumulated glycinebetaine, in particular in their chloroplast, in response to salt stress (Robinson and Jones 1986). It was also shown that 1 M glycinebetaine stabilizes the oxygen-evolving complex of photosystem II by preventing the dissociation of extrinsic proteins and the disintegration of the manganese cluster (Papageorgiou and

Murata 1995). It is likely that glycinebetaine not only acts as an osmoprotectant but also plays essential roles in the protection of the photosynthetic machinery against the effects of high salt. However, when the cells of strain PAMCOD were transferred from NaCl-free medium to medium containing 0.4 M NaCl, their photosynthetic activity declined for two days (Fig. 4-3), and then the photosynthetic activity increased subsequently and the cells started to proliferate again. These effects suggest that some lag time is necessary before the effectiveness of glycinebetaine in protecting the cells against the salt-induced inactivation of photosynthesis becomes apparent.

Changes in tolerance to low temperature

The effect of transformation with the *codA* gene on the ability of the photosynthetic machinery to tolerate low temperatures can be summarized as follows: (1) the transformation enhanced the growth of the cells at low temperature of 20°C; (2) the transformation enhanced the tolerance to low-temperature stress in darkness at temperature from 0 to 10°C; and (3) the transformation enhanced the tolerance to low-temperature stress in light (photoinhibition) at the low temperature of 20°C.

Tolerance to low temperature in darkness

It was previously shown that the inactivation of photosynthesis at low temperature in darkness in *Synechococcus* is caused by the transition of lipid phase of plasma membrane from the liquid-crystalline state to the phase-separated state (Murata 1989). Since changes in absorption spectrum of carotenoid in intact cells are good

indicators of changes in lipid phase in *Synechococcus*, the temperature-dependent profiles of the lipid phase of plasma membrane in the PAM and PAMCOD cells could be determined. The results demonstrated that the phase transition of the plasma membranes of the PAMCOD cells was shifted toward lower temperatures as compared with that of the PAM cells (Fig. 4-11). Since transformation with the *codA* gene did not alter the fatty-acid composition or the major protein components of the PAMCOD cells, it seems likely that the enhancement of the low-temperature tolerance of the photosynthetic machinery in the PAMCOD cells was related to the action *in vivo* of the accumulated glycinebetaine. In artificial membrane systems, it has been demonstrated that glycinebetaine decreases the temperature of the phase transition from the liquid-crystalline state to the gel state (Rudolph et al. 1986, Rudolph and Goins 1991). Thus, it can be proposed that the presence of glycinebetaine in the cytoplasm enhanced the tolerance to low temperature of the cyanobacterial cells by decreasing the temperature for the phase transition.

Tolerance to low temperature in the light

The photoinhibition of photosynthesis *in vivo* is more distinct at low temperature than at normal growth temperatures because the rate of recovery from photoinhibition is remarkably reduced at low temperature (Gombos et al., 1992 and 1994, Kanervo et al., 1995). Therefore, the rate of recovery determines the sensitivity to low-temperature stress. The extent of photoinhibition *in vivo* results from the balance between two processes, the initial damage to D1 protein and the subsequent repair of the

photosystem II complex by incorporation of newly synthesized D1 protein (Aro et al. 1990 and 1993). The results in the present study demonstrated that the photosystem II of the PAMCOD cells was more tolerant to photoinhibition than that of the PAM cells (Fig. 4-8A). The presence of lincomycin reduced the low-temperature tolerance of photosystem II in the PAMCOD cells (Fig. 4-8B), suggesting that it is the recovery process that is accelerated in these latter cells. Direct measurements of the restoration of photosystem II activity (Fig. 4-9) supported this suggestion. These findings indicate that the depression of growth of the PAM cells at 20°C was initially caused by low-temperature photoinhibition, and that glycinebetaine, accumulated in the cytoplasm, sustained the growth of the PAMCOD cells at 20°C by accelerating the recovery process.

Tolerance to high temperature

The effect of transformation with the *codA* gene on the tolerance of photosynthesis to high temperatures can be summarized as follows: (1) the transformation enhanced the growth of the cells at the high temperature of 42°C; (2) the transformation did not affect the tolerance to high-temperature stress in darkness; (3) the transformation did not affect the tolerance to high-temperature stress in the light (photoinhibition).

It was previously shown that the oxygen-evolving photosystem II complex is stabilized to a remarkable extent by 1 M glycinebetaine (Mohanty et al. 1993, Murata et al. 1992, Papageorgiou and Murata 1995, Papageorgiou et al. 1991). Moreover, the stability of the photosystem II complex

at high temperatures in darkness is also enhanced by 1 M glycinebetaine (Mamedov et al. 1991). The author expected, therefore, that the accumulation of glycinebetaine in the PAMCOD cells would stabilize the oxygen-evolving activity at high temperatures. However, the accumulated glycinebetaine had no effect on the stability at high temperatures (Fig. 4-5). The tolerance of photosynthesis to high temperature in the light (photoinhibition) was unaffected by the transformation with the *codA* gene and, therefore, by the accumulated glycinebetaine (Fig. 4-7). It seems likely that the concentration of accumulated glycinebetaine, about 80 mM, was not sufficiently high to be effective in the stabilization of the photosystem II complex. These results suggest that an as yet unidentified effect of glycinebetaine is involved in the enhancement of growth at high temperature in the PAMCOD cells.

References

- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J.D. (1989) *Molecular Biology of the cell*, 2nd Edition. Garland Publishing, Inc. New York. pp. 792-836.
- Andresen, P.A., Kaasen, I., Styrvold, O.B., Boulnois, G. and Strøm, A.R. (1988) Molecular cloning, physical mapping and expression of the *bet* genes governing the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. *J Gen Microbiol.* 134: 1737-1746.
- Apte, S.K. and Bhagwat, A.A. (1989) Salinity-stress-induced proteins in two nitrogen-fixing *Anabaena* strains differentially tolerant to salt. *J Bacteriol.* 171: 909-915.
- Apte, S.K. and Thomas, J. (1986) Membrane electrogenesis and sodium transport in filamentous nitrogen-fixing cyanobacteria. *Eur J Biochem.* 154: 395-401.
- Arnon, D.I., McSwain, B.D., Tsujimoto, H.Y. and Wada K. (1974) Photochemical activity and components of membrane preparations from blue-green algae I. Coexistence of two photosystems in relation to chlorophyll a and removal of phycocyanin. *Biochim Biophys Acta.* 357: 231-245.
- Aro, E-M., Hundal, T., Carlberg, I. and Andersson, B. (1990) *In vitro* studies on light-induced inhibition of photosystem II and D1-protein degradation at low temperature. *Biochim Biophys Acta.* 1019: 269-275.
- Aro, E-M., Virgin, I. and Andersson, B. (1993) Photoinhibition of photosystem II. Inactivation, protein damage and turnover. *Biochim Biophys Acta.* 1143: 113-134.

- Bakker, E.P., Booth, I.R., Dinnbier, U., Epstein, W. and Gajewska, A. (1987) Evidence for multiple K⁺ export systems in *Escherichia coli*. *J Bacteriol.* 169: 3743-3749.
- Bernard, T., Ayache, M. and Rudulier, D.L. (1988) Restoration, by glycine betaine, of growth and enzyme activities of *Escherichia coli* Lac-mutants *C R Acad Sci III.* 307: 99-104.
- Berry, J. and Bjorkman, O. (1980) Photosynthetic response and adaptation to temperature in higher plants. *Annu Rev Plant Physiol.* 31: 491-543.
- Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* 37: 911-917.
- Blomberg, A. (1995) Global changes in protein synthesis during adaptation of the yeast *Saccharomyces cerevisiae* to 0.7 M NaCl. *J Bacteriol.* 177: 3563-3572.
- Blumwald, E., Mehlhorn, R.J. and Packer, L. (1983a) Studies of osmoregulation in salt adaptation of cyanobacteria with ESR spin-probe technique. *Proc Natl Acad Sci USA* 80: 2599-2602.
- Blumwald, E., Mehlhorn, R.J. and Packer, L. (1983b) Ionic osmoregulation during salt adaptation of the cyanobacterium *Synechococcus* 6311. *Plant Physiol.* 73: 377-380.
- Blumwald, E., Wolosin, J.M. and Packer, L. (1984) Na⁺/H⁺ exchange in the cyanobacterium *Synechococcus* 6311. *Biochem Biophys Res Comm.* 122: 452-459.
- Boch, J., Kempf, B. and Bremer, E. (1994) Osmoregulation in *Bacillus subtilis*: synthesis of the osmoprotectant

- glycine betaine from exogenously provided choline. *J Bacteriol.* 176: 5364-5371.
- Bohnert, H.J., Nelson, D.E. and Jensen, R.G. (1995) Adaptations to environmental stresses. *Plant cell* 7: 1099-1111.
- Boos, W., Ehmann, U., Forkl, H., Klein, W., Rimmele, M. and Postma, P. (1990) Trehalose transport and metabolism in *Escherichia coli*. *J Bacteriol.* 172: 3450-3461.
- Brouquisse, R., Weigel, P., Rhodes, D., Yocum, C.F. and Hanson, A.D. (1989) Evidence for a ferredoxin-dependent choline monooxygenase from spinach chloroplast stroma. *Plant Physiol.* 90: 322-329.
- Bukau, B. (1993) Regulation of the *Escherichia coli* heat-shock response. *Mol Microbiol.* 9: 671-680.
- Burg, M.B. (1994) Molecular basis for osmoregulation of organic osmolytes in renal medullary cells. *J Exp Zool.* 268: 171-175.
- Cairney, J., Booth, I.R. and Higgins, C.F. (1985) Osmoregulation of gene expression in *Salmonella typhimurium*: *proU* encodes an osmotically induced betaine transport system. *J Bacteriol.* 164: 1224-1232.
- Carmel, O., Dover, N., Rahav-Manor, O., Dibrov, P., Kirsch, D., Karpel, R., Schuldiner, S. and Padan, E. (1994) A single amino acid substitution (Glu134 → Ala) in Nha1 increases the inducibility by Na⁺ of the product of *nhaA*, a Na⁺/H⁺ antiporter gene in *Escherichia coli*. *EMBO J.* 13: 1981-1989.

- Catevelli, L. and Bartels, D. (1990) Molecular cloning and characterization of cold-regulated genes in barley. *Plant Physiol.* 93: 1504-1510.
- Chauvin, L-P., Houde, M. and Sarhan, F. (1993) A leaf-specific gene stimulated by light during wheat acclimation to low temperature. *Plant Mol Biol.* 23: 255-265.
- Claes, B., Dekeyser, R., Villarroel, R., Van den Bulcke, M., Bauw, G., Van Montagu, M. and Caplan, A. (1990) Characterization of a rice gene showing organ-specific expression in response to salt stress and drought. *Plant Cell* 2: 19-27.
- Csonka, L.N. (1989) Physiological and genetic responses of bacteria to osmotic stress. *Microbiol Rev.* 53: 121-147.
- Csonka, L.N. and Hanson, A.D. (1991) Prokaryotic osmoregulation: genetics and physiology. *Annu Rev Microbiol.* 45: 569-606.
- Curtain, C.C., Looney, F.D., Regan, D.L. and Ivancic, N.M. (1983) Changes in the ordering of lipids in the membrane of *Dunaliella* in response to osmotic-pressure changes an e.s.r. study. *Biochem J.* 213: 131-136.
- Dattananda, C.S. and Gowrishankar, J. (1989) Osmoregulation in *Escherichia coli*: complementation analysis and gene-protein relationships in the *proU* locus. *J Bacteriol.* 171: 1915-1922.
- Dattananda, C.S., Rajkumari, K. and Gowrishankar, J. (1991) Multiple mechanisms contribute to osmotic inducibility of *proU* operon expression in *Escherichia coli*: demonstration of two osmoresponsive promoters and of a negative

- regulatory element within the first structural gene. *J Bacteriol.* 173: 7481-7490.
- Delauney, A.J., Hu, C-A.A., Kavi Kishor, P.B. and Verma, D.P.S. (1993) Cloning of ornithine δ -aminotransferase cDNA from *Vigna aconifolia* by trans-complementation in *Escherichia coli* and regulation of proline biosynthesis. *J Biol Chem.* 268: 18673-18678.
- Delauney, A.J. and Verma, D.P.S. (1993) Proline biosynthesis and osmoregulation in plants. *Plant J.* 4: 215-223.
- Deshnium, P., Los, D.A., Hayashi, H., Mustardy, L. and Murata, N. (1995) Transformation of *Synechococcus* with a gene for choline oxidase enhances tolerance to salt stress. *Plant Mol Biol.* 29: 897-907.
- Deutch, A.H., Smith, C.J., Rushlow, K.E. and Kretschmer, P.J. (1982) *Escherichia coli* Δ^1 -pyrroline-5-carboxylate reductase: gene sequence, protein overproduction and purification. *Nucleic Acids Res.* 10: 7701-7714.
- Deutch, A.H., Smith, C.J., Rushlow, K.E. and Kretschmer, P.J. (1984) Analysis of *Escherichia coli* proBA locus by DNA and protein sequencing. *Nucleic Acids Res.* 12: 6337-6355.
- Einspahr, K.J., Maeda, M. and Thompson, G.A., Jr. (1988) Concurrent changes in *Dunaliella salina* ultrastructure and membrane phospholipid metabolism after hyperosmotic shock. *J Cell Biol.* 107: 529-538.
- Einspahr, K.J., Peeler, T.C. and Thompson, G.A., Jr. (1988) Rapid changes in polyphosphoinositide metabolism associated with the response of *Dunaliella salina* to hypoosmotic shock. *J Biol Chem.* 263: 5775-5779.

- Elhai, J., Thiel, T. and Pakrasi, H.B. (1991) DNA transfer into cyanobacteria. In Plant Molecular Biology Manual. Edited by Gelvin, S. B., Schilperoort, R.A. and Verma, D.P.S. pp. A12: 1-23. Kluwer Academic Publishers, Dordrecht, Netherland.
- Epstein, W. and Kim, B.S. (1971) Potassium transport loci in *Escherichia*. J Bacteriol. 108: 639-644.
- Falkenberg, P. and Strøm, A.R. (1990) Purification and characterization of osmoregulatory betaine aldehyde dehydrogenase of *Escherichia coli*. Biochim Biophys Acta 1034: 253-259.
- Ferrando, A., Kron, S.J., Rios, G., Fink, G.R., and Serrano, R. (1995) Regulation of cation transport in *Saccharomyces cerevisiae* by the salt tolerance gene *HAL3*. Mol Cell Biol. 15: 5470-5481.
- Fry, I.V., Huflejt, M., Erber, W.W, Peschek, G.A. and Packer, L. (1986) The role of respiration during adaptation of the freshwater cyanobacterium *Synechococcus* 6311 to salinity. Arch Biochem Biophys. 244: 686-691.
- Gabbay-Azaria, R., Schonfeld, M, Tel-Or, S., Messinger, R. and Tel-Or, E. (1992) Respiratory activity in the marine cyanobacterium *Spirulina subsalsa* and its role in salt tolerance. Arch Microbiol. 157: 183-190.
- Gabbay-Azaria, R., Tel-Or, E. and Schonfeld, M. (1988) Glycinebetaine as an osmoregulant and compatible solute in the marine cyanobacterium *Spirulina subsalsa*. Arch Biochem Biophys. 264: 333-339 (1988).
- Gaber, R.F., Styles, C.A. and Fink, G.R. (1988) *TRK1* encodes a plasma membrane protein required for high-affinity

- potassium transport in *Saccharomyces cerevisiae*. *Mol Cell Biol.* 8: 2848-2859.
- Garciadeblas, B., Rubio, F., Quintero, F.J., Banuelos, M.A., Haro, R. and Rodriguez-Navarro, A. (1993) Differential expression of two genes encoding isoforms of the ATPase involved in sodium efflux in *Sacharomyces cerevisiae*. *Mol Gen Genet.* 236: 363-368.
- Garcia-Perez, A. and Burg, M.B. (1991) Renal medullary organic osmolytes. *Physiol Rev.* 71: 1081-1115.
- Gaxiola, R., de Larrinoa, I.F., Villalba, J.M. and Serrano, R. (1992) A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. *EMBO J.* 11: 3157-3164.
- Gilmour, S.J., Artus, N.N. and Thomashow, M.F. (1992) cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. *Plant Mol Biol.* 18: 13-21.
- Giæver, H.M., Styrvold, O.B., Kaasen, I. and Strøm A.R. (1988) Biochemical and genetic characterization of osmoregulatory trehalose synthesis in *Escherichia coli*. *J Bacteriol.* 170: 2841-2849.
- Goddard, N.J., Dunn, M.A., Zhang, L., White, A.J., Jack, P.L. and Hughes, M.A. (1993) Molecular analysis and spatial expression pattern of a low-temperature-specific barley gene, *blt101*. *Plant Mol Biol.* 23: 871-879.
- Goldberg, E.B., Arbel, T., Chen, J., Karpel, R., Mackie, G.A., Schuldiner, S. and Padan, E. (1987) Characterization of a Na^+/H^+ antiporter gene of *Escherichia coli*. *Proc Natl Acad Sci USA* 84: 2615-2619.

- Gombos, Z. and Vigh, L. (1986) Primary role of the cytoplasmic membrane in thermal acclimation evidenced in nitrate-starved cells of the blue-green alga, *Anacystis nidulans*. *Plant Physiol* 80: 415-419.
- Gombos, Z., Wada, H. and Murata, N. (1991) Direct evaluation of effects of fatty-acid unsaturation on the thermal properties of photosynthetic activities, as studied by mutation and transformation of *Synechocystis* PCC6803. *Plant Cell Physiol*. 32: 205-211.
- Gombos, Z., Wada, H. and Murata, N. (1992) Unsaturation of fatty acids in membrane lipids enhances tolerance of the cyanobacterium *Synechocystis* PCC6803 to low-temperature photoinhibition. *Proc Natl Acad Sci USA* 89: 9959-9963.
- Gombos, Z., Wada, H. and Murata, N. (1994) The recovery of photosynthesis from low-temperature photoinhibition is accelerated by the unsaturation of membrane lipids: a mechanism of chilling tolerance. *Proc Natl Sci USA* 91: 8787-8791.
- Gowrishankar, J. (1989) Nucleotide sequence of the osmoregulatory *proU* operon of *Escherichia coli*. *J Bacteriol*. 171: 1923-1931.
- Gowrishankar, J., Jayashree, P. and Rajkumari, K. (1986) Molecular cloning of an osmoregulatory locus in *Escherichia coli*: increased *proU* gene dosage results in enhanced osmotolerance. *J Bacteriol*. 168: 1197-1204.
- Grossman, E.B. and Hebert, S.C. (1989) Renal inner medullary choline dehydrogenase activity: characterization and modulation. *Am J Physiol*. 256: F107-F112.

- Grothe, S., Krogsrad, R.L., McClellan, D.J., Milner, J.L. and Wood, J.M. (1986) Proline transport and osmotic stress response in *Escherichia coli* K-12. *J Bacteriol.* 166: 253-259.
- Haardt, M., Kempf, B., Faatz, E. and Bremer, E. (1995) The osmoprotectant proline betaine is a major substrate for the binding-protein-dependent transport system ProU of *Escherichia coli* K-12. *Mol Gen Genet.* 246: 783-786.
- Haest, C.W.H., De Gier, J.A.F., Op den Kamp, J.A.E., Bartels, P. and van Deenen, L.L.M. (1972) Changes in permeability of *Staphylococcus aureus* and derived liposomes with varying lipid composition. *Biochim Biophys Acta* 255: 720-733.
- Hanna, K., Bengis-Garber, C., Kushner, D.J., Kogut, M. and Kates, M. (1984) The effect of salt concentration on the phospholipid and fatty acid composition of the moderate halophile *Vibrio costicola*. *Can J Biochem.* 30: 669-675.
- Hanson, A.D., May, A.M., Grumet, R., Bode, J., Jamieson, G.C. and Rhodes, D. (1985) Betaine synthesis in chenopods: localization in chloroplasts. *Proc Natl Acad Sci USA* 82: 3678-3682.
- Hanson, A.D., Rathinasarapathi, B., Rivoval, J., Burnet, M., Dillon, M.O. and Gage, D.A. (1994) Osmoprotective compounds in the Plumbaginaceae: A natural experiment in metabolic engineering of stress tolerance. *Proc Natl Acad Sci USA* 91: 306-310.
- Hengge-Aronis, R., Klein, W., Lange, R., Rimmele, M. and Boos, W. (1991) Trehalose synthesis genes are controlled by the putative sigma factor encoded by *rhoS* and are involved

- in stationary-phase thermotolerance in *Escherichia coli*. *J Bacteriol.* 173: 7918-7924.
- Holmstrom, K-O, Welin, B., Mandal, A., Kristiansdottir, I., Teeri, T.H., Lamark, T., Strøm, A.R. and Palva, E.T. (1994) Production of the *Escherichia coli* betaine-aldehyde dehydrogenase, an enzyme required for the synthesis of the osmoprotectant glycine betaine, in transgenic plants. *Plant J.* 6: 749-758.
- Houde, M., Danyluk, J., Laliberte, J-F., Rassart, E., Dhindsa, R.S. and Sarhan, F. (1992) Cloning, characterization, and expression of a cDNA encoding a 50-kilodalton protein specifically induced by cold acclimation in wheat. *Plant Physiol.* 99: 1381-1387.
- Hu, C.A., Delauney, A.J. and Verma, D.P. (1992) A bifunctional enzyme (δ 1-pyrroline-5-carboxylate synthetase) catalyzes the first two steps in proline biosynthesis in plants. *Proc Natl Acad Sci USA* 89: 9354-9358.
- Ikuta, S., Matuura, K., Imamura, S., Misaki, H. and Horiuti, Y. (1977a) Oxidative pathway of choline to betaine in the soluble fraction prepared from *Arthrobacter globiformis*. *J Biochem.* 82: 157-163.
- Ikuta, S., Imamura, S., Misaki, H. and Horiuti, Y. (1977b) Purification and characterization of choline oxidase from *Arthrobacter globiformis*. *J Biochem.* 82: 1741-1749.
- Incharoensakdi, A., Takabe, T. and Akazawa, T. (1986) Effect of betaine on enzyme activity and subunit interaction of ribulose-1,5-bisphosphate carboxylase/oxygenase from *Aphanothece halophytica*. *Plant Physiol.* 81: 1044-1049.

- Ishitani, M., Nakamura, T., Han, S.Y. and Takabe, T. (1995) Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid. *Plant Mol Biol.* 27: 307-315.
- Ivey, D.M., Guffanti, A.A., Bossewitch, J.S., Padan, E. and Krulwich, T.A. (1991) Molecular cloning and sequencing of a gene from alkaliphilic *Bacillus firmus* OF4 that functionally complements an *Escherichia coli* strain carrying a deletion in the *nhaA* Na⁺/H⁺ antiporter gene. *J Biol Chem.* 266: 23483-23489.
- Ivey, D.M., Guffanti, A.A., Shen, Z., Kudyan, N. and Krulwich, T.A. (1992) The *cadC* gene product of alkaliphilic *Bacillus firmus* OF4 partially restores Na⁺ resistance to an *Escherichia coli* strain lacking an Na⁺/H⁺ antiporter (NhaA). *J Bacteriol.* 174: 4878-4884.
- Jeanjean, R., Matthijs, H.C.P., Onana, B., Havaux, M. and Joset, F. (1993) Exposure of the cyanobacterium *Synechocystis* PCC6803 to salt stress induces concerted changes in respiration and photosynthesis. *Plant Cell Physiol.* 34: 1073-1079.
- Jia, Z., McCullough, N., Martel, R., Hemmingsen, S. and Young, P. (1992) Gene amplification at a locus encoding a putative Na⁺/H⁺ antiporter confers sodium and lithium tolerance in fission yeast. *EMBO J.* 11: 1631-1640.
- Jones, G.P., Naidu, B.P., Starr, R.K. and Paleg, L.G. (1986) Estimates of solutes accumulating in plants by ¹H nuclear magnetic resonance spectroscopy. *Aust J Plant Physiol.* 13: 649-658.

- Kaenjak, A., Graham, J.E., Wilkinson, B.J. (1993) Choline transport activity in *Staphylococcus aureus* induced by osmotic stress and low phosphate concentrations. *J Bacteriol.* 175: 2400-2406.
- Kagan, R.M. and Clarke, S. (1994) Widespread occurrence of three sequence motifs in diverse S-adenosylmethionine-dependent methyltransferases suggests a common structure for these enzymes. *Arch Biochem Biophys.* 310: 417-427.
- Kanervo, E., Aro, E-M., Murata, N. (1995) Low unsaturation level of thylakoid membrane lipids limits turnover of the D1 protein of photosystem II at high irradiance. *FEBS Lett.* 364: 239-242.
- Karpel, R., Olami, Y., Taglich, D., Schuldiner, S. and Padan, E. (1988) Sequencing of the gene *ant* which affects the Na⁺/H⁺ antiporter activity in *Escherichia coli*. *J Biol Chem.* 263: 10408-10414.
- Kates, M. (1986) Influence of salt concentration on membrane lipids of halophilic bacteria. *FEMS Microbiol Rev.* 39: 95-101.
- Kempf, B. and Bremer, E. (1995) OpuA, an osmotically regulated binding protein-dependent transport system for the osmoprotectant glycine betaine in *Bacillus subtilis*. *J Biol Chem.* 270: 16701-16713.
- Khaware, R.K., Koul, A. and Prasad, R. (1995) High membrane fluidity is related to NaCl stress in *Candida membranefaciens*. *Biochem Mol Biol Int.* 35: 875-880.
- Khomutov, G., Fry, I.V., Huflejt, M.E. and Packer, L. (1990) Membrane lipid composition, fluidity, and surface charge changes in response to growth of the fresh water

- cyanobacterium *Synechococcus* 6311 under high salinity. *Arch Biochem Biophys.* 277: 263-267.
- Kirst, G.O. (1989) Salinity tolerance of eukaryotic marine algae. *Annu Rev Plant Physiol Plant Mol Biol.* 40: 21-53.
- Kishor, P.B.K., Hong, Z., Miao, G-H., Hu, C-A.A. and Verma, D.P.S. (1995) Overexpression of Δ^1 -pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol.* 108: 1387-1394.
- Ko, C.H. and Gaber, R.F. (1991) *TRK1* and *TRK2* encode structurally related K^+ transporters in *Saccharomyces cerevisiae*. *Mol Cell Biol.* 11: 4266-4273.
- Ko, R., Tombras, L. and Smith, G.M. (1994) Glycine betaine confers enhanced osmotolerance and cryotolerance on *Listeria monocytogenes*. *J Bacteriol.* 176: 426-431.
- Koch, A.L. (1984) Shrinkage of growing *Escherichia coli* cells by osmotic stress. *J Bacteriol.* 159: 919-924.
- Kruger, E., Volker, U. and Hecker, M. (1994) Stress induction of *clpC* in *Bacillus subtilis* and its involvement in stress tolerance. *J Bacteriol.* 176: 3360-3367.
- Kunin, C.M., Hua, T.H., Van Arsdale White, L. and Villarejo, M. (1992) Growth of *Escherichia coli* in human urine: role of salt tolerance and accumulation of glycine betaine. *J Infect Dis.* 166: 1311-1315.
- Kurkela, S. and Franck, M. (1990) Cloning and characterization of a cold- and ABA-inducible *Arabidopsis* gene. *Plant Mol Biol.* 15: 137-144.
- Lamark, T., Kaasen, I., Eshoo, M.W., Falkenberg, P., McDougall, J. and Strøm, A.R. (1991) DNA sequence and

- analysis of the *bet* genes encoding the osmoregulatory choline-glycine betaine pathway of *Escherichia coli*. *Mol Microbiol.* 5: 1049-1064.
- Landfald, B. and Strøm, A.R. (1986) Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. *J Bacteriol.* 165: 849-855.
- Lang, V. and Palva, E.T. (1992) The expression of a rab-related gene, *rab18*, is induced by abscisic acid during the cold-acclimation process of *Arabidopsis thaliana* (L.) Heynh. *Plant Mol Biol.* 20: 951-962.
- Ledeboer, A.M., Edens, L., Maat, J., Visser, C., Bos, J.W., Verrips, C.T., Janowicz, Z., Eckart, M., Roggenkamp, R. and Hollenberg, C.P. (1985) Molecular cloning and characterization of a gene coding for methanol oxidase in *Hansenula polymorpha*. *Nucl Acids Res.* 13: 3063-3082.
- Lewis, M.J. and Pelham, H.R.B. (1985) Involvement of ATP in the nuclear and nucleolar functions of the 70 kD heat shock protein. *EMBO J.* 4: 3137-3143.
- Liberek, K. and Georgopoulos, C. (1993) Autoregulation of the *Escherichia coli* heat shock response by the DnaK and DnaJ heat shock proteins. *Proc Natl Acad Sci USA* 90: 11019-11023.
- Lucht, J.M., Dersch, P., Kempf, B. and Bremer, E. (1994) Interactions of the nucleoid-associated DNA-binding protein H-NS with the regulatory region of the osmotically controlled *proU* operon of *Escherichia coli*. *J Biol Chem.* 269: 6578-6586.
- Lyons, J.M. (1973) Chilling injury in plants. *Annu Rev Plant Physiol.* 24: 445-466.

- Mackie, G.A. (1986) Structure of the DNA distal to the gene for ribosomal protein S20 in *Escherichia coli* K12: presence of a strong terminator and an IS1 element. *Nucleic Acids Res.* 14: 6965-6981.
- Maeda, M. and Thompson, G.A., Jr. (1986) On the mechanism of rapid plasma membrane and chloroplast envelope expansion in *Dunaliella salina* exposed to hypoosmotic shock. *J Cell Biol.* 102: 289-297.
- Mager, W.H. and de Kruijff, A.J.J. (1995) Stress-induced transcriptional activation. *Microbiol Rev.* 59: 506-531.
- Mamedov, M.D., Hayashi, H., Wada, H., Mohanty, P.S., Papageorgiou, G.C. and Murata, N. (1991) Glycinebetaine enhances and stabilizes the evolution of oxygen and the synthesis of ATP by cyanobacterial thylakoid membranes. *FEBS Lett.* 294: 271-274.
- Matin, A. (1991) The molecular basis of carbon-starvation-induced general resistance in *Escherichia coli*. *Mol Microbiol.* 5: 3-10.
- McCue, K.F. and Hanson, A.D. (1990) Drought and salt tolerance: towards understanding and application. *Trends Biotechnol.* 8: 358-362.
- McCue, K.F. and Hanson, A.D. (1992) Salt-inducible betaine aldehyde dehydrogenase from sugar beet: cDNA cloning and expression. *Plant Mol Biol.* 18: 1-11.
- Memon, A.R., Herrin, D.L. and Thompson, G.A., Jr. (1993) Intracellular translocation of a 28 kDa GTP-binding protein during osmotic shock-induced cell volume regulation in *Dunaliella salina*. *Biochim Biophys Acta* 1179: 11-22.

- Meury, J. and Kepes, A. (1982) Glutathione and gated potassium channels of *Escherichia coli*. *EMBO J.* 1: 339-343.
- Meury, J., Robin, A. and Monnier-Champeix, P. (1985) Turgor-controlled K^+ fluxes and their pathways in *Escherichia coli*. *Eur J Biochem.* 151: 613-619.
- Miller, K.J. (1985) Effects of temperature and sodium chloride concentration on the phospholipid and fatty acid compositions of a halotolerant *Planococcus* sp. *J Bacteriol.* 162: 263-270.
- Miyake, C., Michihata, F. and Asada, K. (1991) Scavenging of hydrogen peroxide in prokaryotic and eukaryotic algae: acquisition of ascorbate peroxidase during the evolution of cyanobacteria. *Plant Cell Physiol.* 32: 33-43.
- Mohanty, P., Hayashi, H., Papageorgiou, G.C. and Murata, N. (1993) Stabilization of the Mn-cluster of the oxygen-evolving complex by glycinebetaine. *Biochim Biophys Acta* 1144: 92-96.
- Molitor, V., Walter, E. and Pescheck, G.A. (1986) Increased levels of cytochrome oxidase and sodium-proton antiporter in the plasma membrane of *Anacystis nidulans* after growth in sodium-enriched media. *FEBS Lett.* 204: 251-256.
- Murata, N., Ishizaki-Nishizawa, O., Higashi, S., Hayashi, H., Tasaka, Y. and Nishida, I. (1992) Genetically engineered alteration in the chilling sensitivity of plants. *Nature* 356: 710-713.
- Murata, N. (1989) Low-temperature effects on cyanobacterial membranes. *J Bioenerg Biomembr.* 21: 61-75.
- Murata, N., Mohanty, P.S., Hayashi, H. and Papageorgiou, G.C. (1992) Glycinebetaine stabilizes the association of

- extrinsic proteins with the photosynthetic oxygen-evolving complex. *FEBS Lett.* 296: 187-189.
- Murata, N., Sato, N., Takahashi, N. and Hamazaki, Y. (1982) Compositions and positional distributions of fatty acids in phospholipids from leaves of chilling-sensitive and chilling-resistant plants. *Plant Cell Physiol.* 23: 1071-1079.
- Murata, N. and Wada, H. (1995) Acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria. *Biochem J.* 308: 1-8.
- Nishiyama, Y., Hidenori, H., Watanabe, T. and Murata, N. (1994) Photosynthetic oxygen evolution is stabilized by cytochrome C₅₅₀ against heat inactivation in *Synechococcus* sp. PCC 7002. *Plant Physiol.* 105: 1313-1319.
- Nishiyama, Y., Kovacs, E., Lee, C.B., Hayashi, H., Watanabe, T. and Murata, N. (1993) Photosynthetic adaptation to high temperature associated with thylakoid membranes of *Synechococcus* PCC7002. *Plant Cell Physiol.* 34: 337-343.
- Nitschmann, W.H. and Packer, L. (1992) NMR studies on Na⁺ transport in *Synechococcus* PCC 6311. *Arch Biochem Biophys.* 294: 347-352.
- Nomura, M., Ishitani, M., Takabe, T., Rai, A.K. and Takabe, T. (1995) *Synechococcus* sp. PCC 7942 transformed with *Escherichia coli bet* genes produces glycine betaine from choline and acquires resistance to salt stress. *Plant Physiol.* 107: 703-708.
- Nordin, K., Vahala, T. and Palva, E.T. (1993) Differential expression of two related, low-temperature-induced genes

- in *Arabidopsis thaliana* (L.) Heynh. *Plant Mol Biol.* 21: 641-653.
- Ohishi, N. and Yagi, K. (1979) Covalently bound flavin as prosthetic group of choline oxidase. *Biochem Biophys Res Commun.* 86: 1084-1088.
- Omata, T. and Murata, N. (1983) Isolation and characterization of the cytoplasmic membranes from the blue-green alga (cyanobacterium) *Anacystis nidulans*. *Plant Cell Physiol.* 24: 1101-1112.
- Ono, T. and Murata, N. (1981) Chilling susceptibility of the blue-green alga *Anacystis nidulans* I. Effect of growth temperature. *Plant Physiol* 67: 176-181.
- Orr, W., Iu, B., White, T.C., Robert, L.S. and Singh, J. (1992) Complementary sequence of a low temperature-induced *B. napus* gene with homology to the *A. thaliana kin1* gene. *Plant Physiol.* 98: 1532-1534.
- Overdier, D.G. and Csonka, L.N. (1992) A transcriptional silencer downstream of the promoter in the osmotically controlled *proU* operon of *Salmonella typhimurium*. *Proc Natl Acad Sci USA* 89: 3140-3144.
- Packer, L., Spath, S., Martin, J.B., Roby, C. and Bligny, R. (1987) ²³Na and ³¹P NMR studies of the effects of salt stress on the freshwater cyanobacterium *Synechococcus* 6311. *Arch Biochem Biophys.* 256: 354-361.
- Padan, E. and Schuldiner, S. (1994a) Molecular physiology of the Na⁺/H⁺ antiporter in *Escherichia coli*. *J exp Biol.* 196: 443-456.
- Padan, E. and Schuldiner, S. (1994b) Molecular biology of Na⁺/H⁺ antiporters: molecular devices that couple the Na⁺

- and H⁺ circulation in cells. *Biochim Biophys Acta* 1187: 206-210.
- Padan, E. and Schuldiner, S. (1994c) Molecular physiology of Na⁺/H⁺ antiporters, key transporters in circulation of Na⁺ and H⁺ in cells. *Biochim Biophys Acta* 1185: 129-151.
- Papageorgiou, G.C., Fujimura, Y. and Murata, N. (1991) Protection of the oxygen-evolving photosystem II complex by glycinebetaine. *Biochim Biophys Acta* 1057: 361-366.
- Papageorgiou, G.C. and Murata, N. (1995) The unusually strong stabilizing effects of glycine betaine on the structure and function in the oxygen-evolving photosystem II complex. *Photosynth Res.* 44: 243-252.
- Paschinger, H. (1977) Sodium chloride stimulated respiration of *Anacystis nidulans*. *Z Allg Mikrobiol.* 17: 377-379.
- Paul, M. (1990) Limited N-terminal sequence analysis. *Meth Enzymol.* 182:602-613.
- Pinner, E., Kotler, Y., Padan, E. and Schuldiner, S. (1993) Physiological role of NhaB, a specific Na⁺/H⁺ antiporter in *Escherichia coli*. *J Biol Chem.* 268: 1729-1734.
- Pinner, E., Padan, E. and Schuldiner, S. (1992) Cloning, sequencing and expression of *nhaB* gene, encoding a Na⁺/H⁺ antiporter in *Escherichia coli*. *J Biol Chem.* 267: 11064-11068.
- Pinner, E., Padan, E. and Schuldiner, S. (1994) Kinetic properties of NhaB, a Na⁺/H⁺ antiporter from *Escherichia coli*. *J Biol Chem.* 269: 26274-26279.
- Pocard, J-A., Smith, L.T., Smith, G.M. and Le Rudulier, D. (1994) A prominent role for glucosylglycerol in the

- adaptation of *Pseudomonas mendocina* SKB70 to osmotic stress. *J Bacteriol.* 176: 6877-6884.
- Polarek, J.W., Walderhaug, M.O. and Epstein, W. (1987) Genetics of Kdp, the K⁺ transport ATPase of *Escherichia coli*. *Meth Enzymol.* 157: 655-667.
- Raison, J.K. (1973) The influence of temperature-induced phase changes on kinetics of respiratory and other membrane-associated enzymes. *J Bioenerg.* 4: 258-309.
- Ramirez, R.M., Prince, W.S., Bremer, E. and Villarejo, M. (1989) *In vitro* reconstitution of osmoregulated expression of *proU* of *Escherichia coli*. *Proc Natl Acad Sci USA* 86: 1153-1157.
- Rahav-Manor, O., Carmel, O., Karpel, R., Taglicht, D., Glaser, G., Schuldiner, S. and Padan, E. (1992) NhaR, a protein homologous to a family of bacterial regulatory proteins (LysR) regulates *nhaA*, the sodium proton antiporter gene in *Escherichia coli*. *J Biol Chem.* 267: 10433-10438.
- Ratzkin, B., Grabnar, M. and Roth, J. (1978) Regulation of the major proline permease in *Salmonella typhimurium*. *J Bacteriol.* 133: 737-743.
- Repoila, F. and Gutierrez, C. (1991) Osmotic induction of the periplasmic trehalose in *Escherichia coli* K12: characterization of the *treA* gene promoter. *Mol Microbiol.* 5: 747-755.
- Riethdorf, S., Volker, U., Gerth, U., Winkler, A., Engelmann, S. and Hecker, M. (1994) Cloning, nucleotide sequence, and expression of the *Bacillus subtilis lon* gene. *J Bacteriol.* 176: 6518-6527.

- Riviere, M.E., Arrio, B., Steffan, I., Molitor, V., Kuntner, O. and Peschek, G.A. (1990) Changes of some physical properties of isolated and purified plasma and thylakoid membrane vesicles from the freshwater cyanobacterium *Synechococcus* 6301 (*Anacystis nidulans*) during adaptation to salinity. *Arch Biochem Biophys.* 280: 159-166.
- Rhodes, D., Handa, S. and Bressan, R.A. (1986) Metabolic changes associated with adaptation of plant cells to water stress. *Plant Physiol.* 82: 890-903.
- Rhodes, D. and Hanson, A.D. (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annu Rev Plant Physiol Plant Mol Biol.* 44: 357-384.
- Robinson, S.P. and Jones, G.P. (1986) Accumulation of glycinebetaine in chloroplasts provides osmotic adjustment during salt stress. *Aust J Plant Physiol.* 13: 659-668.
- Rozwadowski, K.L., Khachatourians, G.G. and Selvaraj, G. (1991) Choline oxidase, a catabolic enzyme in *Arthrobacter pascens*, facilitates adaptation to osmotic stress in *Escherichia coli*. *J Bacteriol.* 173: 472-478.
- Rudolph, A.S., Crowe, J.H. and Crowe, L.M. (1986) Effects of three stabilizing agents -proline, betaine, and trehalose- on membrane phospholipids. *Arch Biochem Biophys* 245: 134-143.
- Rudolph, A.S. and Goins, B. (1991) The effect of hydration stress solutes on the phase behavior of hydrated dipalmitoylphosphatidylcholine. *Biochim Biophys Acta* 1066: 90-94.
- Russell, N.J., Adams, R., Bygraves, J. and Kogut, M. (1986) Cell envelope phospholipid changes in a moderate halophile

- during phenotypic adaptation to altered salinity and osmotic stress. *FEMS Microbiol Rev.* 39: 103-107.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning. a laboratory manual.* Cold Spring Harbor, New York, USA.
- Santoro, M.M., Liu, Y., Khan, S.M., Hou, L.X. and Bolen, D.W. (1992) Increased thermal stability of proteins in the presence of naturally occurring osmolytes. *Biochemistry* 31: 5278-5283.
- Sato, N. (1994) A cold-regulated cyanobacterial gene cluster encodes RNA-binding protein and ribosomal protein S21. *Plant Mol Biol.* 24: 819-823.
- Sato, N. (1995) A family of cold-regulated RNA-binding protein genes in the cyanobacterium *Anabaena variabilis* M3. *Nucleic Acids Res.* 23: 2161-2167.
- Sato, N. and Murata, N. (1980) Temperature shift-induced response in lipids in the blue-green alga, *Anabaena variabilis*. The central role of diacylmonogalactosylglycerol in thermo-adaptation. *Biochim Biophys Acta* 619: 353-366.
- Sato, N. and Murata, N. (1981) Studies on the temperature shift-induced desaturation of fatty acid in monogalactosyl diacylglycerol in the blue-green alga (cyanobacterium), *Anabaena variabilis*. *Plant Cell Physiol.* 22: 1043-1050.
- Sato, N. and Murata, N. (1988) Membrane lipids. *Meth Enzymol.* 167: 251-259.
- Schobert, B. and Tschesche, H. (1978) Unusual solution properties of proline and its interaction with proteins. *Biochim Biophys Acta* 541: 270-277.

- Shigeoka, S., Takeda, T., Ishikawa, T. and Mutsuda, M. (1995) Enzymatic and molecular characterization of catalase-peroxidase in *Synechococcus* PCC 7942. In *Photosynthesis: from light to biosphere*. Edited by Mathis, P. V: 143-146. Kluwer Academic Publishers, Netherland.
- Siebers, A., Wieczorek, L. and Altendorf, K. (1987) K⁺-ATPase from *Escherichia coli*: isolation and characterization. *Meth Enzymol.* 157: 668-680.
- Smirnoff, N. and Cumbes, Q.J. (1989) Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry* 28: 1057-1060.
- Stanier, R.Y., Kunisawa, R., Mandel, M. and Cohen-Bazier, G. (1971) Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriol Rev.* 35: 171-205.
- Strøm, A.R. and Kaasen, I. (1993) Trehalose metabolism in *Escherichia coli*: stress protection and stress regulation of gene expression. *Mol Microbiol.* 8: 205-210.
- Styrvold, O.B. and Strøm, A.R. (1991) Synthesis, accumulation, and excretion of trehalose in osmotically stressed *Escherichia coli* K-12 strains: influence of amber suppressors and function of the periplasmic trehalose. *J Bacteriol.* 173: 1187-1192.
- Uchida, S., Kwon, H.M., Yamauchi, A., Preston, A.S., Marumo, F. and Handler, J.S. (1992) Molecular cloning of the cDNA for an MDCK cell Na⁺ and Cl⁻-dependent taurine transporter that is regulated by tonicity. *Proc Natl Acad Sci.* 89: 8230-8234.

- Uchida, S., Yamauchi, A., Preston, A.S., Kwon, H.M. and Handler, J.S. (1993) Transcription of the gene for the betaine transporter in MDCK cells is stimulated by hypertonicity. *J Clin Invest.* 91: 1604-1607.
- Verbruggen, N., Villarroel, R. and Van Montagu, M. (1993) Osmoregulation of a pyrroline-5-carboxylate reductase gene in *Arabidopsis thaliana*. *Plant Physiol.* 103: 771-781.
- Voelker, U., Voelker, A., Maul, B., Hecker, M., Dufour, A. and Haldenwang, W.G. (1995) Separate mechanisms activate σ^B of *Bacillus subtilis* in response to environmental and metabolic stresses. *J Bacteriol.* 177: 3771-3780.
- Wall, J.S., Christianson, D.D., Dimler, R.J. and Senti, F.R. (1960) Spectrophotometric determination of betaines and other quaternary nitrogen compounds as their periodides. *Analyt Chem.* 32: 870-874.
- Wada, H., Gombos, Z. and Murata, N. (1990) Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation. *Nature* 347: 200-203.
- Wada, H., Gombos, Z. and Murata, N. (1994) Contribution of membrane lipids to the ability of the photosynthetic machinery to tolerate temperature stress. *Proc Natl Acad Sci USA* 91: 4273-4277.
- Wada, H. and Murata, N. (1989) *Synechocystis* PCC6803 mutants defective in desaturation of fatty acids. *Plant Cell Physiol.* 30: 971-978.
- Weigel, P., Weretilnyk, E.A. and Hanson, A.D. (1986) Betaine aldehyde oxidation by spinach chloroplasts. *Plant Physiol.* 82: 753-759.

- Weretilnyk, E.A., Bednarek, S., McCue, K.F., Rhodes, D. and Hanson, A.D. (1989) Comparative biochemical and immunological studies of the glycine betaine synthesis pathway in diverse families of dicotyledons. *Planta* 178: 342-352.
- Weretilnyk, E.A. and Hanson, A.D. (1990) Molecular cloning of a plant betaine-aldehyde dehydrogenase, an enzyme implicated in adaptation to salinity and drought. *Proc Natl Acad Sci USA* 87: 2745-2749.
- Weretilnyk, E., Orr, W., White, T.C., Iu, B. and Singh, J. (1993) Characterization of three related low temperature-regulated cDNAs from winter *Brassica napus*. *Plant Physiol.* 101: 171-177.
- White, T.C., Simmonds, D., Donaldson, P. and Singh, J. (1994) Regulation of *BN115*, a low-temperature-responsive gene from winter *Brassica napus*. *Plant Physiol.* 106: 917-928.
- Wierenga, R.K., Terpstra, P. and Hol, W.G.J. (1986) Prediction of the occurrence of the ADP-binding $\beta\alpha\beta$ -fold in proteins, using an amino acid sequence fingerprint. *J Mol Biol.* 187: 101-107.
- Winzor, C.L., Winzor, D.J., Paleg, L.G., Jones, G.P. and Naidu, B.P. (1992) Rationalization of the effects of compatible solutes on protein stability in terms of thermodynamic nonideality. *Arch Biochem Biophys.* 296: 102-107.
- Wolfraim, L.A. and Dhindsa, R.S. (1993) Cloning and sequencing of the cDNA for *cas17*, a cold acclimation-specific gene of alfalfa. *Plant Physiol.* 103: 667-668.

- Wolfrain, L.A., Langis, R., Tyson, H. and Dhindsa, R.S.
(1993) cDNA sequence, expression, and transcript stability of a cold acclimation-specific gene, *cas18*, of alfalfa (*Medicago falcata*) cells. *Plant Physiol.* 101: 1275-1282.
- Wood, D., Darlison, M.G., Wilde, R.J. and Guest, J.R. (1984) Nucleotide sequence encoding the flavoprotein and hydrophobic subunits of the succinate dehydrogenase of *Escherichia coli*. *Biochem J.* 222: 519-534.
- Wood, J.M. (1988) Proline porters affect the utilization of proline as a nutrient or osmoprotectant for bacteria. *J Membrane Biol.* 106: 183-202.
- Yamauchi, A., Uchida, S., Kwon, H.M., Preston, A.S., Robey, R.B., Garcia-Perez, A., Burg, M.B. and Handler, J.S.
(1992) Cloning of a Na⁺- and Cl⁻-dependent betaine transporter that is regulated by hypertonicity. *J Biol Chem.* 267: 649-652.
- Yancy, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D. and Somero, G.N. (1982) Living with water stress: evolution of the osmolyte systems. *Science* 217: 1214-1222.
- Zhu, J-K. Shi, J., Singh, U., Wyatt, S.E., Bressan, R.A., Hasegawa, P.M. and Carpita, N.C. (1993) Enrichment of vitronectin- and fibronectin-like proteins in NaCl-adapted plant cells and evidence for their involvement in plasma membrane-cell wall adhesion. *Plant J.* 3: 637-646.