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PsbU, a Protein Associated with Photosystem II, Is Required for the Acquisition of Cellular Thermotolerance in *Synechococcus* species PCC 7002[1]

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PsbU is an extrinsic protein of the photosystem II complex of cyanobacteria and red algae. Our previous in vitro studies (Y. Nishiyama, D.A. Los, H. Hayashi, N. Murata [1997] Plant Physiol 115: 1473–1480) revealed that PsbU stabilizes the oxygen-evolving machinery of the photosystem II complex against heat-induced inactivation in the cyanobacterium *Synechococcus* sp. PCC 7002. To elucidate the role of PsbU in vivo, we inactivated the *psbU* gene in *Synechococcus* sp. PCC 7002 by targeted mutagenesis. Inactivation of the *psbU* gene resulted in marked changes in the acclimative responses of cells to high temperature: Mutated cells were unable to increase the thermal stability of their oxygen-evolving machinery when grown at moderately high temperatures. Moreover, the cellular thermotolerance of the mutated cells failed to increase upon acclimation of cells to high temperature. The heat-shock response, as assessed in terms of the levels of homologs of the heat-shock proteins Hsp60, Hsp70, and Hsp17, was unaffected by the mutation in *psbU*, suggesting that heat-shock proteins were not involved in the changes in the acclimative responses. Our observations indicate that PsbU is involved in the mechanism that underlies the enhancement of the thermal stability of the oxygen-evolving machinery and that the stabilization of the oxygen-evolving machinery is crucial for the acquisition of cellular thermotolerance.

Photosynthetic organisms can modify their photosynthesis machinery in response to changes in ambient temperature. When such organisms have become acclimated to moderately high temperatures, their photosynthesis machinery exhibits enhanced thermal stability (Berry and Björkman, 1980). This acclimative response has been observed in several species of plants (Armond et al., 1978; Pearcy, 1978; Raison et al., 1982) and cyanobacteria (Lehel et al., 1993; Nishiyama et al., 1993).

High-temperature stress causes the irreversible inactivation of the PSII complex, a pigment-protein complex in which light energy is used to drive the transport of electrons and the oxidation of water to molecular oxygen. Of the various components of the photosynthesis machinery, the PSII complex is often the most susceptible to high temperature (Berry and Björkman, 1980; Mamedov et al., 1993). Several studies have demonstrated that the heat-induced inactivation of the PSII complex is initiated by the destruction of the manganese cluster that catalyzes the oxidation of water to oxygen (Nash et al., 1985; Thompson et al., 1989; Mamedov et al., 1993). Thus, the thermal stability of the oxygen-evolving machinery of the PSII complex appears to influence the thermal stability of the entire photosynthesis system. We reported previously that it is only the oxygen-evolving site that is stabilized in the PSII complex during acclimation to high temperature in the cyanobacterium *Synechococcus* sp. PCC 7002 (Nishiyama et al., 1993). All of these observations together indicate that enhancement of the thermal stability of the oxygen-evolving machinery might be an important acclimative response in cells that are able to tolerate elevated temperatures.

A number of investigators have tried to define factors that stabilize the PSII complex against heat-induced inactivation. It has been suggested that Hsps (Stapel et al., 1993; Eriksson and Clarke, 1996; Heckathorn et al., 1998), carotenoids of the xanthophyll cycle (Havaux et al., 1996), and isoprene (Sharkey and Singsaas, 1995) might protect the PSII complex against heat stress. However, it remains unclear whether these factors are involved in the enhancement of thermal stability of the PSII complex during acclimation to high temperatures. Some researchers correlated the thermal stability of the PSII complex with levels of saturated membrane lipids in terms of acclimation to high temperature (Pearcy, 1978; Raison et al., 1982; Thomas et al., 1986). However, studies of mutant cyanobacteria defective in the desaturation of fatty acids provided direct evidence that contradicted previous suggestions that saturated lipids might be important in the thermal stability of the oxygen-evolving machinery (Gombos et al., 1991, 1994; Mamedov et al., 1993; Wada et al., 1994; Moon et al., 1995). The results of these studies implicated factors other than membrane lipids in the enhancement of the thermal stability of the oxygen-evolving machinery during acclimation to high temperatures.

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Abbreviations: Chl, chlorophyll; Hsp, heat-shock protein; Sp^r, spectinomycin resistance.

The oxygen-evolving activity of thylakoid membranes isolated from cells of *Synechococcus* sp. PCC 7002 grown at high temperatures exhibited greater thermal stability than that from cells grown at low temperatures (Nishiyama et al., 1993). This suggested that factors responsible for thermal stability might be associated with thylakoid membranes. Biochemical investigations of thylakoid membranes allowed us to identify two proteins, Cyt c_{550} and PsbU, as factors that stabilize the oxygen-evolving machinery against heat-induced inactivation (Nishiyama et al., 1994, 1997). Cyt c_{550} and PsbU are the extrinsic proteins of PSII complexes that have been found in several species of cyanobacteria (Stewart et al., 1985; Shen et al., 1997, 1998) and red algae (Enami et al., 1995), but they are not found in higher plants (Shen and Inoue, 1993). These proteins are expressed constitutively regardless of growth temperature, and neither undergoes any modification during the acclimation of cells to high temperatures (Nishiyama et al., 1997). These characteristics led us to conclude that Cyt c_{550} and PsbU are the components of the PSII complex that constitutively stabilize the oxygen-evolving system, not the factor(s) that directly modifies the thermal stability of the oxygen-evolving machinery during acclimation to high temperature.

In the present study we inactivated the *psbU* gene in *Synechococcus* sp. PCC 7002 by targeted mutagenesis to examine the role of PsbU in vivo, in particular, as it relates to acclimation to high temperatures. Mutated cells were no longer able to increase the thermal stability of the oxygen-evolving machinery and were also unable to develop cellular thermotolerance upon acclimation to high temperature.

MATERIALS AND METHODS

Organism and Culture Conditions

The wild-type strain of *Synechococcus* sp. PCC 7002 was obtained from the Culture Collection of the Pasteur Institute (Paris). Cells were grown photoautotrophically at 25°C or 38°C for 3 to 5 d in medium A, as described by Stevens et al. (1973), under a light intensity of 70 μmol m^{-2} s^{-1} and with aeration by sterile air containing 1% CO_2. The growth of cells was monitored in terms of turbidity at 730 nm with an absorption spectrophotometer (model UV300, Shimadzu, Kyoto, Japan).

Targeted Mutagenesis

Plasmid pUH239 (Nishiyama et al., 1997), which carries the *psbU* gene, was partially digested with *Ecl*136II. An Sp^r gene cartridge was obtained from plasmid pBS-Sp^r (Los et al., 1997) by digestion with *Hinc*II and *Ecl*136II, and this cartridge was ligated into the partially digested pUH239. The resultant plasmids were used to transform *Escherichia coli* JM109, and the spectinomycin-resistant clones of *E. coli* were isolated. The correct insertion of the Sp^r gene cartridge was determined by restriction analysis. The final construct contained the *psbU* gene with insertion of the Sp^r gene cartridge in the same orientation. This plasmid was designated pBSU::Sp^r and was used for transformation of wild-type cells of *Synechococcus* sp. PCC 7002 using the method described by Williams (1988).

Assays of the Thermal Stability of the Oxygen-Evolving Machinery and Cellular Thermotolerance

To examine the thermal stability of the oxygen-evolving machinery, we incubated cells at a density of 5 to 7 μg Chl mL^{-1} at designated temperatures for 20 min in darkness. After incubation, each suspension of cells was promptly cooled to 30°C, and the oxygen-evolving activity was measured. Cellular thermotolerance was examined by monitoring the viability of cells as follows. Cells were grown on plates of agar-solidified medium A at 25°C or 38°C for 10 to 14 d under a light intensity of 70 μmol m^{-2} s^{-1}. The plates were then transferred to 43°C and incubated for 2 d under the same intensity of light. The viability of cells was determined from visible damage such as bleaching. Cellular thermotolerance was also examined by monitoring the viability of cells after exposure to a lethal high temperature. Cells in liquid medium at a density of 5 to 7 μg Chl mL^{-1} were incubated at 49°C for various periods in darkness, and then cultures were promptly cooled to 30°C. Aliquots were diluted 10,000-fold and spread on plates of agar-solidified medium A. The plates were incubated at 30°C for 2 weeks under a light intensity of 70 μmol m^{-2} s^{-1}, and then viable colonies were counted.

Measurements of Photosynthesis Activity

The rate of photosynthesis evolution of oxygen was determined by monitoring the concentration of oxygen in a suspension of cells with a Clark-type oxygen electrode. Net photosynthesis activity of cells was measured at 30°C in medium A without any exogenously added electron acceptor. PSII activity in cells was measured at 30°C in medium A that had been supplemented with 1 mM 1,4-benzoquinone. Red actinic light at an intensity of 2 mmol m^{-2} s^{-1} was provided by an incandescent lamp in conjunction with heat-absorbing (HA50, Hoya, Tokyo) and red (R-60, Toshiba, Tokyo) optical filters. Concentrations of Chl were determined as described by Arnon et al. (1974).

Analysis of Hsps

Levels of homologs of Hsp60, Hsp70, and Hsp17 in cells were determined by western analysis. Cells were harvested by centrifugation at 8000*g* for 10 min and washed with 50 mM Hepes-NaOH (pH 7.5) containing 30 mM $CaCl_2$. The following procedures were performed at 0°C to 4°C. The sedimented cells were suspended in 50 mM Hepes-NaOH (pH 7.5) containing 800 mM sorbitol, 5 mM $MgCl_2$, 1 mM 6-amino-*n*-caproic acid, 1 mM benzamidine hydrochloride, and 1 mM PMSF. The suspension was homogenized with an equal volume of glass beads (diameter, 0.1 mm) on a vortex mixer (Vortex Genie-2, Scientific Industries, Bohemia, NY) operated at maximum speed for 3 min with 30 s of rest every 30 s. The homogenate was centrifuged at 5000*g* for 10 min to remove unbroken cells and cell debris,

and the supernatant that contained soluble and membrane components was collected.

Aliquots equivalent to 10 μg of protein were subjected to electrophoresis on a 10% polyacrylamide gel for the analysis of homologs of Hsp60 and Hsp70 and on a 17% polyacrylamide gel for the analysis of the homolog of Hsp17. The separated proteins were blotted onto a PVDF membrane (Clear Blot, Atto, Tokyo) and allowed to react with antisera that had been raised in rabbits against Hsp60 and Hsp70 of *Synechococcus vulcanus*, both of which were prepared as a mixture of multiple isoforms (Tanaka et al., 1997), and with an antiserum that had been raised in rats against Hsp17 of *Synechocystis* sp. PCC 6803 (H. Fukuzawa, H. Kosaka, K. Lee, and K. Ohyama, unpublished data). Concentrations of proteins were determined with a protein assay solution (Bio-Rad) with BSA as the standard according to the method described by Bradford (1976).

RESULTS

Targeted Mutagenesis of the *psbU* Gene in *Synechococcus* sp. PCC 7002

The *psbU* gene was inactivated by insertion of the Sp^r gene cartridge at the *Ecl*136II site of the *psbU* gene in wild-type cells of *Synechococcus* sp. PCC 7002 by homologous recombination (Fig. 1A), and the resultant mutant strain was designated *psbU*$^-$. The complete replacement of the native gene by the mutated gene was confirmed by PCR with the primers indicated by arrows in Figure 1A. A DNA fragment of 0.4 kb that originated from the native *psbU* gene was amplified by PCR with the genomic DNA from wild-type cells as the template (Fig. 1B, lane 1). A DNA fragment of 2.4 kb, which was expected to encompass a *psbU* gene that included an inserted Sp^r gene cartridge, was amplified by PCR with the genomic DNA from *psbU*$^-$ cells as the template (Fig. 1B, lane 2). The results indicated that the native gene had been replaced by the mutated gene in every copy of the chromosome in cells of the mutant strain.

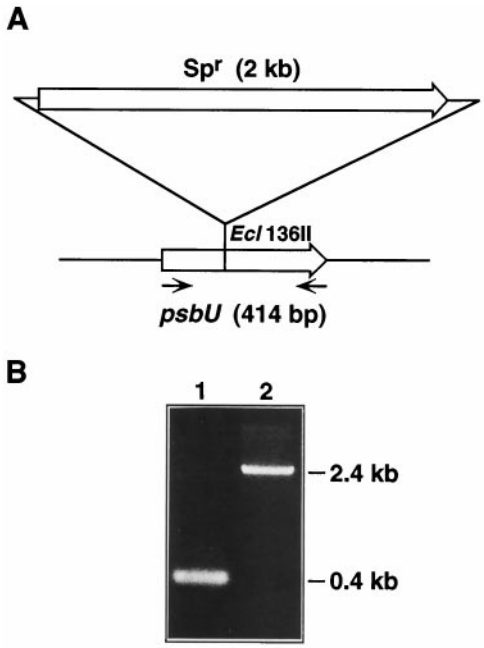

Figure 1. Targeted mutagenesis of the *psbU* gene in the chromosome of *Synechococcus* sp. PCC 7002. A, Construction of the plasmid pBSU::Sp^r in which the *psbU* gene was disrupted by an Sp^r gene cartridge. Small arrows indicate the primers used for PCR for evaluation of the replacement of the native *psbU* gene. B, Analysis by PCR to confirm the complete replacement of the native gene by the mutated gene. Genomic DNA isolated from wild-type (lane 1) and *psbU*$^-$ (lane 2) cells of *Synechococcus* sp. PCC 7002 was used as the template for PCR.

Table I. *Effects of inactivation of the psbU gene on photosynthesis oxygen-evolving activities in Synechococcus sp. PCC 7002 cells*

PSII activity was measured by monitoring the evolution of oxygen in the presence of 1 mM 1,4-benzoquinone. The net photosynthesis activity was measured by monitoring the evolution of oxygen without exogenously added electron acceptors. Values are means ± SE of results from three independent experiments.

Reaction	Growth Temperature	Evolution of O_2 at 30°C	
		Wild type	*psbU*$^-$
	°C	μmol O_2 mg^{-1} Chl h^{-1}	
PSII	25	879 ± 115	800 ± 92
	38	638 ± 62	593 ± 44
Net photosynthesis	25	356 ± 36	342 ± 14
	38	151 ± 27	137 ± 3

Changes in Photosynthesis Activities

The photosynthesis activities, measured in terms of the evolution of oxygen at 30°C, in wild-type and *psbU*$^-$ cells are shown in Table I. Cells were grown at a moderately low temperature (25°C) and a moderately high temperature (38°C) to examine the dependence on growth temperature. After the cells grew at 25°C, PSII activity was not significantly different between wild-type and *psbU*$^-$ cells when the evolution of oxygen was measured in the presence of 1,4-benzoquinone as the electron acceptor. A similar tendency was observed when cells were grown at 38°C. The net photosynthesis activity measured in the absence of exogenous electron acceptors was not significantly different between the two types of cell. These observations suggested that depletion of PsbU did not have a significant effect on the oxygen-evolving activity when cells were grown at temperatures within the normal physiological range. Thus, PsbU was not essential for the catalytic activities required for the evolution of oxygen.

Changes in the Thermal Stability of the Oxygen-Evolving Machinery

We examined the thermal stability of the oxygen-evolving machinery in wild-type and *psbU*$^-$ cells that had been grown at 25°C or at 38°C. As we reported previously (Nishiyama et al., 1993), the growth temperature had a considerable effect on the thermal stability of the PSII activity of wild-type cells: In cells that had been grown at 38°C, the oxygen-evolving activity exhibited greater thermal stability than that in cells that had been grown at 25°C

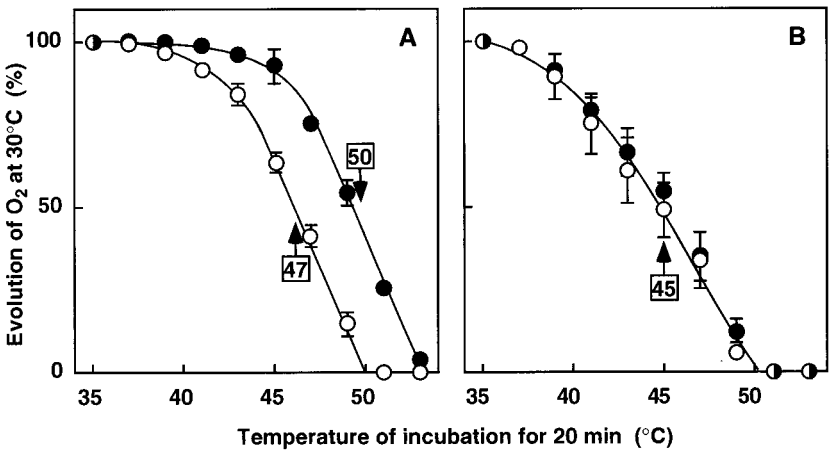

Figure 2. Profiles of the inactivation by heat of the oxygen-evolving machinery of wild-type (A) and *psbU*$^-$ (B) cells of *Synechococcus* sp. PCC 7002. Cells were cultivated at 25°C (○) and 38°C (●). Cells at a density of 5 to 7 μg Chl mL^{-1} were incubated at the designated temperatures in darkness for 20 min, and the evolution of oxygen was measured at 30°C in the presence of 1 mM 1,4-benzoquinone. The oxygen-evolving activities taken as 100% were 879, 638, 800, and 593 μmol O_2 mg^{-1} Chl h^{-1} in wild-type cells grown at 25°C, in wild-type cells grown at 38°C, in *psbU*$^-$ cells grown at 25°C, and in *psbU*$^-$ cells grown at 38°C, respectively. The numbers in boxes refer to temperatures (°C) for 50% inactivation. Values are means ± SE (bars) of results from three independent experiments.

(Fig. 2A). The temperature for 50% inactivation shifted from 47°C to 50°C when the growth temperature was increased from 25°C to 38°C. Thus, in wild-type cells, the thermal stability of the oxygen-evolving machinery increased upon the acclimation of cells to a high temperature. In contrast, the thermal stability of the oxygen-evolving activity in *psbU*$^-$ cells did not increase when the growth temperature was increased from 25°C to 38°C (Fig. 2B). The temperature for 50% inactivation was 45°C in *psbU*$^-$ cells grown at 25°C and in *psbU*$^-$ cells grown at 38°C, and it was lower by 2°C than the temperature for 50% inactivation of wild-type cells grown at 25°C. Essentially the same results were obtained when the thermal stability of net photosynthesis was examined (data not shown). Thus, the absence of PsbU resulted not only in a decrease in the thermal stability of the oxygen-evolving machinery but also in loss of the ability to increase this stability. These findings suggested that PsbU might be involved in the mechanism that underlies the increase in thermal stability of the oxygen-evolving machinery that occurs during the acclimation of cells to high temperatures.

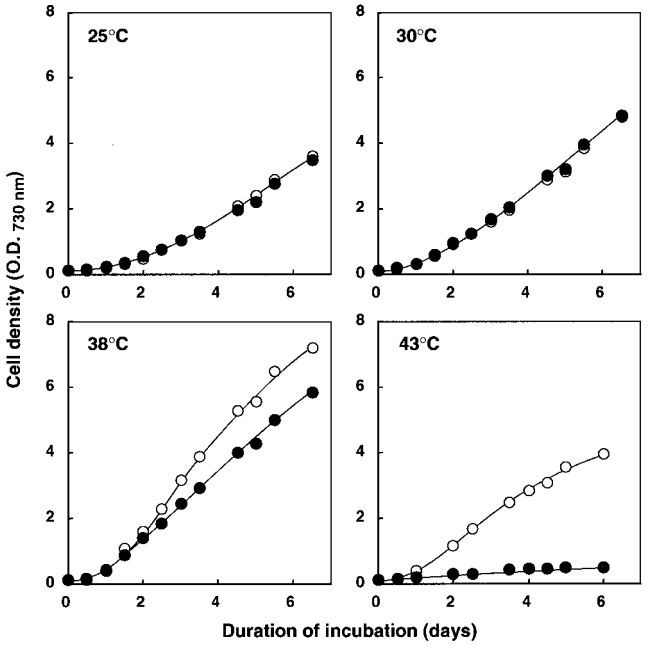

Figure 3. Growth of wild-type and *psbU*$^-$ cells at various temperatures. Cells were cultivated at 35°C, and then cultures were transferred to the indicated temperatures. ○, Wild-type cells; ●, *psbU*$^-$ cells. Values are the averages of results from two independent experiments. O.D., Optical density.

Changes in the Growth of Cells

We examined the effect of the absence of PsbU on the growth of cells at various temperatures (Fig. 3). Wild-type and *psbU*$^-$ cells grew at the same rate at 25°C, a moderately low temperature, and at 30°C, a medium temperature, while the *psbU*$^-$ cells grew slightly slower than wild-type cells at 38°C, a moderately high temperature. Wild-type cells were able to grow at 43°C, a very high temperature, when they were transferred to this temperature after initial growth at 35°C, whereas *psbU*$^-$ cells did not grow after transfer to 43°C. These results indicated that PsbU might be required for cell growth under conditions of high-temperature stress.

Changes in Cellular Thermotolerance

We investigated the role of PsbU in the acquisition of cellular thermotolerance. When wild-type and *psbU*$^-$ cells were grown at 25°C for 14 d on agar plates, they were indistinguishable (Fig. 4A). When agar plates were transferred to 43°C and incubated, bleaching of Chl occurred in both types of cell, and all cells died within 2 d (Fig. 4B). Wild-type and *psbU*$^-$ cells grew well at 38°C (Fig. 4C); however, when agar plates were transferred from 38°C to 43°C, wild-type cells continued to grow but *psbU*$^-$ cells died within 2 d (Fig. 4D). Thus, it seemed likely that wild-type cells had become acclimated to a moderately high temperature during growth at 38°C, and consequently, they were able to survive at 43°C, a temperature that was lethal for nonacclimated cells. *psbU*$^-$ cells did not have any similar ability to acquire cellular thermotolerance.

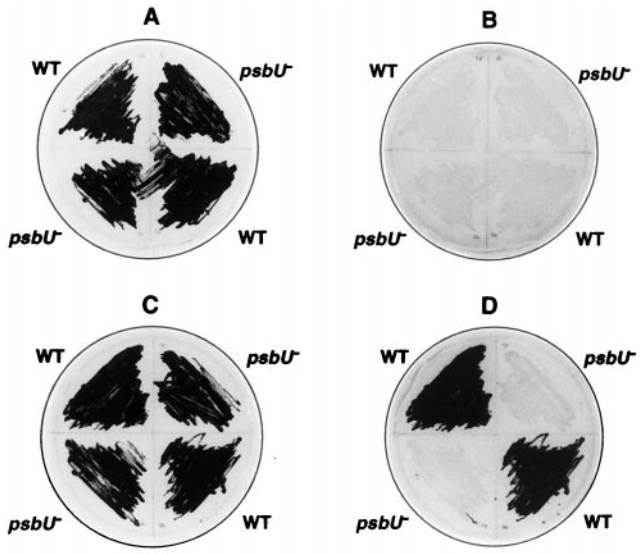

Figure 4. Acquisition of thermotolerance by wild-type (WT) and *psbU*$^-$ cells. Cells were grown at 25°C for 14 d or at 38°C for 10 d on agar plates. The agar plates were then transferred to 43°C and incubated for 2 d. A, Cells grown at 25°C; B, cells transferred to 43°C after growth at 25°C; C, cells grown at 38°C; D, cells transferred to 43°C after growth at 38°C.

For a quantitative analysis of cellular thermotolerance, we examined the viability of wild-type and *psbU*$^-$ cells after exposure of cells to 49°C, a lethal high temperature, for various periods in darkness. All wild-type cells that had been grown at 25°C died after incubation at 49°C for 10 min. In contrast, when wild-type cells had been grown first at 38°C, approximately 80% of cells survived after incubation at 49°C for 10 min (Fig. 5A). All *psbU*$^-$ cells that had been grown at 25°C died after incubation at 49°C for 10 min. When the mutant cells had been grown at 38°C, less than 10% of cells survived after incubation at 49°C for 10 min (Fig. 5B).

Levels of Hsps

One of the conspicuous responses of cells to high temperature is the induction of Hsps (Vierling, 1991; Parsell and Lindquist, 1993). To determine whether the differences in acclimative responses between wild-type and *psbU*$^-$ cells might have been related to the levels of Hsps, we compared levels of homologs of Hsp60, Hsp70, and Hsp17 in wild-type and *psbU*$^-$ cells using western analysis. The basal level of the Hsp60 homolog was the same in both types of cell when cells were grown at 25°C or 38°C (Fig. 6A). The basal level of the Hsp70 homolog was also the same in both types of cell (Fig. 6A). The Hsp17 homolog did not accumulate in wild-type or *psbU*$^-$ cells when they were grown at either 25°C or 38°C, and it accumulated when a heat shock at 43°C was given (Fig. 6A). To examine the induction of the Hsp homologs at a high temperature, we transferred cells that had been grown at 30°C to 43°C and incubated them for various periods without changing the illumination and aeration conditions. The levels of the homologs of Hsp60 and Hsp70 doubled in 3 h in both types of cell, and the pattern of induction was the same as well (Fig. 6B). The Hsp17 homolog accumulated to the same level in both types of cell in 1 h, and the level did not change upon further incubation within 3 h (Fig. 6B). Thus, the basal levels and the heat-shock responses of the Hsp60, Hsp70, and Hsp17 homologs were similar in wild-type and *psbU*$^-$ cells.

DISCUSSION

Role of PsbU in the Oxygen-Evolving Machinery at Moderate Temperatures

In the present study we inactivated the *psbU* gene in *Synechococcus* sp. PCC 7002 by targeted mutagenesis to examine the role of PsbU in vivo. The activity of PSII was not significantly affected by inactivation of the *psbU* gene when cells were grown at moderate temperatures such as 25°C and 38°C. The net photosynthesis activity was also only slightly affected at these temperatures. These results indicate that PsbU is not essential for the catalytic activities required for the evolution of oxygen and, therefore, it has no major effect on the entire photosynthesis system at normal physiological temperatures. This conclusion is supported by our earlier biochemical results showing that the removal and reintegration of PsbU did not significantly

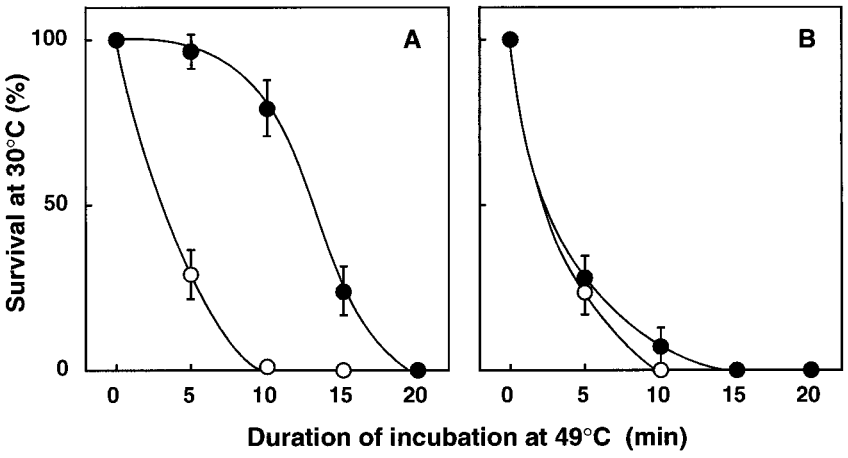

Figure 5. Viability of wild-type (A) and *psbU*$^-$ (B) cells after heat shock. Cells were cultivated at 25°C (○) and 38°C (●). Cells at a density of 5 to 7 μg Chl mL^{-1} were incubated at 49°C for various periods in darkness. Then aliquots were spread on agar plates, and numbers of viable cells were determined. One-hundred percent viability corresponds to the number of viable cells determined in the absence of heat shock. Values are means ± SE (bars) of results from three independent experiments.

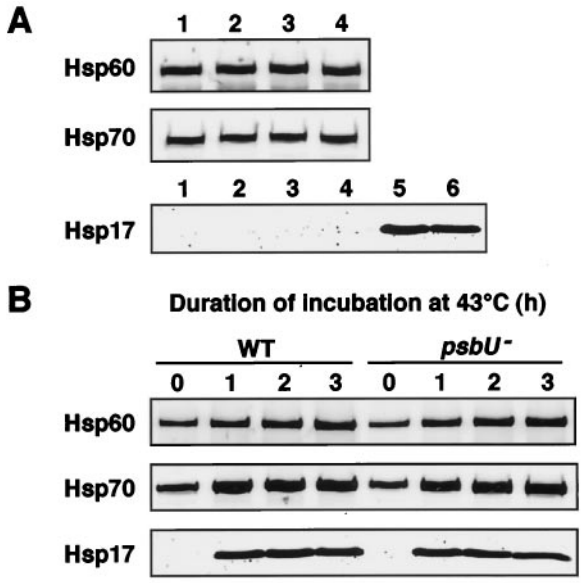

Figure 6. Levels of homologs of Hsp60, Hsp70, and Hsp17 in wild-type (WT) and *psbU*$^-$ cells. Total protein was prepared from cells as described in "Materials and Methods." Levels of homologs of Hsp60, Hsp70, and Hsp17 were determined by western analysis with antisera against Hsp60 and Hsp70 of *S. vulcanus* and with an antiserum against Hsp17 of *Synechocystis* sp. PCC 6803, as described in "Materials and Methods." A, Basal levels of the homologs of Hsp60, Hsp70, and Hsp17 in cells that had been grown at 25°C and 38°C. Lane 1, Wild-type cells grown at 25°C; lane 2, *psbU*$^-$ cells grown at 25°C; lane 3, wild-type cells grown at 38°C; lane 4, *psbU*$^-$ cells grown at 38°C; lanes 5 and 6, levels of the homolog of Hsp17 in wild-type and *psbU*$^-$ cells, respectively, which were incubated at 43°C for 2 h after growth at 30°C. B, Heat-shock response of the homologs of Hsp60, Hsp70, and Hsp17. Cells that had been grown at 30°C were transferred to 43°C and incubated for the designated times.

affect the oxygen-evolving activity of thylakoid membranes from *Synechococcus* sp. PCC 7002 unless the membranes were incubated at high temperatures (Nishiyama et al., 1997). Characterization of a PsbU-deletion mutant of *Synechocystis* sp. PCC 6803 also showed that absence of PsbU had no significant effect on the oxygen-evolving activity and suggested a regulatory role for PsbU in the maintenance of the normal S-state transitions of the oxygen-evolving system (Shen et al., 1997, 1998).

Role of PsbU in Maintenance of the Thermal Stability of the Oxygen-Evolving Machinery

Our previous in vitro studies demonstrated that PsbU, together with Cyt c_{550}, stabilizes the oxygen-evolving machinery against heat-induced inactivation (Nishiyama et al., 1997). In accordance with these results, the present in vivo study showed that inactivation of the *psbU* gene resulted in a decrease in the thermal stability of the oxygen-evolving machinery, indicating that PsbU plays a substantial role in stabilizing this machinery against heat-induced inactivation in vivo.

Upon acclimation of cells of *Synechococcus* sp. PCC 7002 to high temperature, the thermal stability of the oxygen-evolving machinery increases to a significant extent (Nishiyama et al., 1993). Examination of partial electron-transport reactions in the PSII complex revealed that enhancement of thermal stability occurred only at the site of the evolution of oxygen (Nishiyama et al., 1993). Accordingly, direct protection of this site, the catalytic manganese cluster, appears to be the mechanism responsible for acclimation. We initially suspected that expression of the genes for PsbU and Cyt c_{550} might be regulated by temperature. However, biochemical analysis indicated that the level of PsbU was constant regardless of growth temperature and was stoichiometric with respect to the PSII complex. Moreover, the chemical properties of PsbU did not change during acclimation to high temperature, suggesting that modifications such as phosphorylation of PsbU do not occur (Nishiyama et al., 1997). Essentially the same results were obtained for Cyt c_{550} (Y. Nishiyama and N. Murata, unpublished data). These observations led us to conclude that PsbU and Cyt c_{550} are the components of the PSII complex that act constitutively to stabilize the oxygen-evolving machinery and that some undefined factor(s) is required for the increased thermal stability that is related to acclimation. However, we could not rule out the possibility that these proteins might be indirectly involved in enhancing the thermal stability of the oxygen-evolving machinery.

The present study showed that the absence of PsbU resulted in the loss of the ability of cells to increase the thermal stability of their oxygen-evolving machinery upon acclimation to high temperature (Fig. 2), demonstrating clearly that PsbU is involved in the enhancement of thermal stability. Our working model of the mechanism that underlies the enhancement of thermal stability is as follows. During acclimation to high temperature, synthesis of some as-yet-unidentified factor(s) is induced and this factor(s) binds to the donor side of the PSII complex. Presumably, the factor interacts with PsbU and enhances the thermal stability of the oxygen-evolving machinery by strengthening the binding of PsbU to the core of the PSII complex. In the absence of PsbU, the factor cannot bind to the PSII complex and, consequently, it is unable to stabilize the system.

The induction of Hsps often has been correlated with the protection of the PSII complex from heat-induced inactivation (Vierling, 1991; Lehel et al., 1993; Eriksson and Clarke, 1996). In particular, considerable attention has been paid to the role of low-molecular-mass Hsps (Staple et al., 1993; Heckathorn et al., 1998). However, the present study showed that levels of neither the Hsp60 homolog nor the Hsp70 homolog increased when the growth temperature was increased from 25°C to 38°C (Fig. 6), even though the thermal stability of the oxygen-evolving machinery was markedly enhanced (Fig. 2). Moreover, the homolog of Hsp17, a low-molecular-mass Hsp, did not accumulate at all when cells were grown at normal physiological temperatures (at least below 38°C; Fig. 6). In mesophilic cyanobacteria the synthesis of Hsps is, in general, induced at temperatures above 40°C (Borbély et al., 1985; Lehel et al., 1993). Therefore, it seems unlikely that Hsps are involved

in the enhancement of the thermal stability of the oxygen-evolving machinery during acclimation to high temperature.

Role of PsbU in Cellular Thermotolerance

Photosynthesis is a physiological activity in plant cells that is markedly impaired at high temperatures (Berry and Björkman, 1980). The oxygen-evolving machinery is often the most sensitive to high temperature of the various components of the photosynthesis apparatus (Berry and Björkman, 1980; Mamedov et al., 1993). These observations led us to postulate that the thermal sensitivity of the oxygen-evolving machinery might influence the overall tolerance of cells to high temperature. In addition, in view of the acclimation of the photosynthesis machinery to high temperature, we speculated that the enhancement of the thermal stability of the oxygen-evolving machinery might be an important phenomenon that allows increased cellular thermotolerance under high-temperature stress. Our *psbU*$^-$ mutant enabled us to examine these possibilities, since only the thermal stability of the oxygen-evolving machinery was reduced in the mutant cells, and the capacity for enhancement of the thermal stability of the oxygen-evolving machinery was lost. Our examination of thermotolerance revealed that *psbU*$^-$ cells failed to survive at 43°C even after they had been grown at 38°C, a moderately high temperature, whereas wild-type cells were able to survive under the same conditions (Fig. 4). This clearly supports our hypothesis about the role of the acclimation of the photosynthesis system and demonstrates that the stabilization of the oxygen-evolving machinery is crucial for increases in cellular thermotolerance. Our analysis of homologs of Hsp60, Hsp70, and Hsp17 suggested that the decrease in cellular thermotolerance of *psbU*$^-$ cells was not related to Hsps (Fig. 6).

Physiological Implications of the Acclimation of the Photosynthesis Machinery

Our quantitative analysis of the cellular thermotolerance of the *psbU*$^-$ mutant provided more detailed evidence about the role of stabilization of the oxygen-evolving machinery. The inability to enhance the thermal stability of the oxygen-evolving machinery resulted in a considerable reduction in the ability to increase cellular thermotolerance (Fig. 5). When cells are grown at 38°C, some acclimative responses to high temperature other than the acclimation of the photosynthesis machinery, which might be related to thermotolerance, are also likely to occur. However, the effects of such responses on thermotolerance appeared to be suppressed in *psbU*$^-$ cells. In addition, it should be noted that we assayed the viability of cells that had been allowed to grow at 30°C under moderate illumination after exposure to 49°C in darkness (Fig. 5). If the destroyed oxygen-evolving machinery had been repaired by newly synthesized proteins during growth at 30°C, no difference in viability between the two types of cell would have been observed. Thus, the impaired thermotolerance of *psbU*$^-$ cells suggests that, once the oxygen-evolving machinery has been destroyed by heat, the machinery can no longer be repaired. Therefore, it seems probable that the heat-induced destruction of the oxygen-evolving machinery causes lethal damage to the cell. The photosynthetic acclimation that we describe might function to protect the oxygen-evolving machinery from destruction upon a further increase of temperature, which might otherwise lead to cell death. It remains to be determined whether such lethal damage is caused simply by the irreversible destruction of the oxygen-evolving machinery or by toxic oxidative reactants produced from the destroyed machinery.

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