Constitutive expression of a small heat-shock protein confers cellular thermotolerance and thermal protection to the photosynthetic apparatus in cyanobacteria

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Abstract The role of a small heat-shock protein (Hsp) in the acquisition of thermotolerance in cyanobacteria was investigated. *Synechococcus* sp. PCC 7942 was transformed with an expression vector carrying the coding sequence of the *hspA* gene encoding a small heat-shock protein from *Synechococcus vulcanus* under the control of the *tac* promoter. The transformant which was shown to constitutively express HspA displayed improved viability compared with the reference strain upon transfer from 30 to 50°C in the light. When the heat shock was given in darkness, the survival rate in the reference strain increased greatly, approaching a level similar to that for the HspA expressing strain after heat shock in the light. Expression of HspA increased thermal resistance of photosystem II (PS II) and protected phycocyanin from heat-induced photobleaching. Our results are indicative of a central role for HspA in amelioration of the harmful effect of light during heat stress and identified the possible sites of action of the small Hsp in vivo to be the PS II complex and the light-harvesting phycobilisomes. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Cyanobacterium; Small heat-shock protein; Thermotolerance; Photoinhibition; Photosystem; Phycocyanin

1. Introduction

Acquisition of thermotolerance by a cell is correlated with the elevated expression of a set of proteins called heat-shock proteins (Hsps) [1,2]. Among the most prominent Hsps, which have been detected in virtually all types of organisms, the small Hsps or low-molecular-mass Hsps are a group of proteins having molecular masses ranging from 15 to 42 kDa [3,4]. The primary structures of small Hsps are much less conserved across species than those of higher-molecular-mass Hsps such as Hsp70 and Hsp60, although small Hsps have conserved sequences of ~100 amino acids in the C-terminal region that are in common with α-crystallins [3,4]. They form large oligomers with subunits which may vary in number from organism to organism.

The overexpression of small Hsps such as human Hsp27 [5], chinese hamster Hsp27 [6], murine Hsp25 [7], *Drosophila melanogaster* Hsp27 [8], α B-crystallin [9], and bovine eye lens-α A-crystallin [10] have conveyed thermoresistance in mammalian cells, indicating a general thermoprotective function of these small Hsps in mammalian cells. On the other hand, disruption of *hsp26* in *Saccharomyces cerevisiae* did not affect growth characteristics and survival in different culture media at various temperatures or the acquisition of thermotolerance in either log phase or stationary phase cells [11]. Disruption of the *hsp30* gene in *Neurospora crassa* produced a rather complicated phenotype [12]. When high temperature was the sole environmental stress, the mutant strains could survive at high temperatures and acquire thermotolerance from a heat shock as well as the wild type. However, if high temperature was coupled with restricted carbohydrate availability, survival of the *hsp30* mutant strains was reduced by 90%, compared to the wild type. The *ibp* null mutant of *Escherichia coli* which lacks the two small Hsp homologues, IbpA and IbpB, exhibited only a subtle growth defect at 46°C and did not have a reduced cell viability at 50°C [13]. Surprisingly, overproduction of IbpA and IbpB in *E. coli* was rather toxic to cells and led to 5- to 10-fold reductions in cell viability in basal thermotolerance assays at 50°C [13]. To our knowledge, there is no report of a beneficial effect of an overproduction of a prokaryotic small Hsp on thermotolerance. However, heterologous expression of eukaryotic small Hsps enhances *E. coli* viability under heat stress. For example, overexpression of plant cytosolic class I small Hsps from rice and chestnut conferred thermotolerance to *E. coli* [14,15]. Overexpression of murine and human α B-crystallins can also improve *E. coli* thermotolerance [16,17]. Thus, the role of the bacterial small Hsps is still enigmatic.

Cyanobacteria are photoautotrophic prokaryotes that are phylogenetically and physiologically related to chloroplasts of photosynthetic eukaryotes. Thus, cyanobacteria, especially transformable ones such as *Synechocystis* sp. PCC 6803, have been used as model organisms to elucidate chloroplast functions such as photosynthesis. Just like higher plants, small Hsp is one of the most prominent heat-induced proteins in cyanobacteria [18], indicating that small Hsp plays a role in thermal stress management in photosynthetic organisms. Recently, the *hsp16.6* gene, encoding a small Hsp homologue in *Synechocystis* sp. PCC 6803 was inactivated [19]. An increase in heat sensitivity was reflected in changes in growth and oxy-

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Abbreviations: Hsp, heat-shock protein; PS II, photosystem II

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gen evolution rates of the mutant cells after heat shock [19]. The mutants were less viable at high temperatures and lost acquired thermotolerance [20].

In order to understand the role of small Hsps under stress conditions, we have cloned a small Hsp gene from the thermophilic cyanobacterium *Synechococcus vulcanus* [21]. We designated the gene *hspA*. The HspA protein showed high homology (56% identity) with the deduced amino acid sequence of *hsp16.6* from the mesophilic cyanobacterium *Synechocystis* sp. PCC 6803 [18,21]. Purified HspA which formed a large homo-oligomer consisting of 24 subunits prevented thermal aggregation of model substrates, although it could not refold heat-denatured protein [18]. In the present paper, in order to demonstrate the thermoprotective function of the *hspA* gene in vivo and elucidate targets of the small Hsp action in cyanobacteria, we constructed a cyanobacterial strain which expresses HspA constitutively. This is the first attempt to constitutively express any kind of Hsp in cyanobacteria.

2. Materials and methods

2.1. Organisms and culture conditions

Synechococcus sp. PCC 7942 (the small-plasmid-cured derivative of *Anacystis nidulans* R2, R2-SPc [22]) was used throughout. The cells were cultured photoautotrophically in BG-11 inorganic liquid medium [23] or on BG-11 plates containing 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate. The BG-11 was modified to contain 5 mM Tes–NaOH, pH 8.0 and 50 mg/l Na_2CO_3. The liquid culture in a flat glass vessel was continuously aerated. Unless otherwise indicated, cultures were grown at 30°C with a light intensity of 30 μE/m^2/s.

2.2. Construction of the plasmid expressing HspA with a shuttle expression vector

In order to express the *hspA* gene derived from the thermophilic cyanobacterium *S. vulcanus* in the mesophilic cyanobacterium, *Synechococcus* sp. PCC 7942, the *hspA* gene was subcloned into *E. coli–Synechococcus* sp. PCC 7942 shuttle vector pECAN8 [24]. The 0.9-kb *Hin*cII fragment containing the entire coding region of *hspA* including a putative Shine–Dalgarno sequence, but excluding the promoter region [21] was inserted into the *Sma*I site, one of the multiple restriction sites within the *lacZ′* gene of pECAN8. The recombinant plasmid pEC16-1 which carries *hspA* transcribed in the opposite orientation of the *lac* promoter was cut at the *Sal*I multiple restriction site and a 1.3-kb fragment containing the *tac* promoter and a kanamycin resistant cassette (pUC4K, Pharmacia), ligated in opposite orientation, was blunt-ended and inserted into the *Sal*I site. We designated the resultant plasmid pECT16-1 (Fig. 1A). In this construct, *hspA* is under the control of the *tac* promoter. The plasmid, pECT, which was used as a reference plasmid was constructed in the same manner as pECT16-1, but it did not contain the *hspA* gene.

2.3. Transformation of cyanobacteria

Each of the plasmids, pECT and pECT16-1, were introduced into *Synechococcus* sp. PCC 7942 by a method described previously [25]. The cyanobacterial transformants were selected on BG-11 plate supplemented with 20 μg/ml kanamycin.

2.4. Protein analysis in Synechococcus sp. PCC 7942

Cells growing exponentially at 30°C were diluted to an absorbance of 0.1 at 730 nm. Cultures (12 ml) before or after heat-shock treatment were harvested by centrifugation at room temperature for 10 min at 3000 rpm in Beckman GS-6 table-top centrifuge. The cell pellet was collected, and either preserved at −20°C or used immediately. The cells were suspended in 500 μl of 25 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$, and 1 mM each of phenylmethylsulfonyl fluoride, benzamidine and caproic acid. All of the following procedures were carried out at 4°C unless stated otherwise. The cell suspension was mixed with approximately 300 μl volume of glass beads (Sigma) and disrupted by vortexing vigorously for 3 min. The process was repeated three times, and the resulting suspension was centrifuged at less than $1000\times g$ for 5 min. The supernatant which did not contain glass beads was centrifuged again at $16\,000\times g$ for 30 min. The supernatant fraction (designated as a soluble fraction) was kept on ice, and the pellet was washed with 1000 μl of extraction buffer. The suspension was then centrifuged again at $16\,000\times g$ for 30 min, and the pellet was suspended with 500 μl of the extraction buffer. An equal volume of each sample was loaded onto a 15% polyacrylamide gel in the presence of SDS [18]. Immunoblot analysis using anti-*S. vulcanus* HspA, anti-*S. vulcanus* GroEL, or anti-*S. vulcanus* DnaK polyclonal antibodies as probes, and the N-terminal amino acid sequence analysis of a polypeptide were performed as described previously [18].

2.5. Viability assays

The *Synechococcus* sp. PCC 7942 cells were grown to a mid-log phase at 30°C under a light intensity of 30 μE/m^2/s as described above and then diluted to absorbance of 0.1 at 730 nm. For basal thermotolerance assays, 15 ml of the diluted culture was incubated at 50°C for either 15 or 60 min. An aliquot of the culture before or after the high temperature treatment was serially diluted (five times dilution each time) in fresh, sterile BG-11 medium. A 10-μl aliquot from each dilution was then spotted onto duplicate BG-11 plates, and the plates were incubated at 30°C under a light intensity of 30 μE/m^2/s for 1–2 weeks. Cell survival was determined by counting the number of colonies in the most and second most diluted cultures and multiplying its average by the appropriate dilution factor. At least four independent replicate experiments utilizing different cultures were carried out for all temperature experiments. Acquired (or induced) thermotolerance assays were performed as above except 15 ml of the diluted culture was given a pretreatment at 45°C for 60 min prior to the high temperature treatment at 50°C for 60 min. In both basal and acquired thermotolerance assays, the cultures were continuously bubbled with air, and the light intensity was maintained at 30 μE/m^2/s throughout the heat treatments at 45 or 50°C. For the dark incubation, heat shock was performed as above except the culture vessel and the connecting tubes for the aeration were covered completely with aluminum foil.

2.6. Measurements of oxygen evolution

Rates of oxygen evolution were measured with a DW1 oxygen electrode unit (Hansatech Ltd., Norfolk, UK) at 30°C at a photosynthetic photon flux density of 1200 μE/m^2/s. Oxygen evolution was measured either in the presence of 10 mM $NaHCO_3$ and 50 μg/ml streptomycin or 1 mM 1,4-benzoquinone, 1 mM $K_3Fe(CN)_6$ and 50 μg/ml streptomycin.

2.7. Measurements of whole cell absorbance and chlorophyll content

Chlorophyll was determined as described previously [26]. Whole cell absorbance was measured at room temperature with a Hitachi 557 double wavelength double beam spectrophotometer (Hitachi Koki, Tokyo, Japan).

3. Results

3.1. Constitutive expression of the hspA gene from S. vulcanus in Synechococcus sp. PCC 7942

We could not use the parent organism of HspA, *S. vulcanus*, to construct a cyanobacterial strain that constitutively expresses a small Hsp because recombinant techniques are not available for this cyanobacterium. Thus, *Synechococcus* sp. PCC 7942, a mesophilic cyanobacterium, was chosen to express the HspA protein because this cyanobacterium is naturally competent and easily transformable [25]. Furthermore, plasmid vectors to introduce a foreign DNA to *Synechococcus* sp. PCC 7942 are available. Conveniently, there is no homologous small Hsp in *Synechococcus* sp. PCC 7942 that reacts with rabbit polyclonal antibodies against HspA from *S. vulcanus* [18]. Thus, it is possible to detect HspA from *S. vulcanus* specifically in the cyanobacterium using the antibody without a cross-reaction with an endogenous small Hsp in *Synechococcus* sp. PCC 7942.

In order to express the *hspA* gene constitutively in *Synecho-*

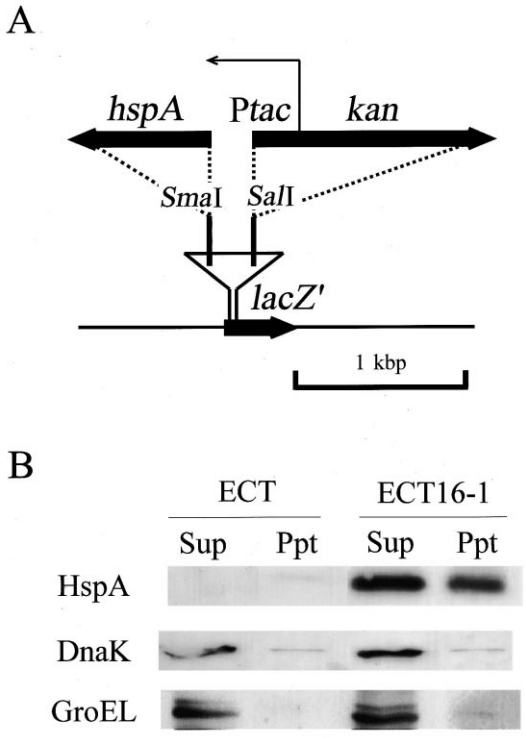

Fig. 1. Constitutive expression of the *hspA* gene. A: Construction of a clone for constitutive expression of *hspA*. Two of the four cloning sites within the *lacZ'* gene in pECAN8, *Sma*I and *Sal*I, were used to clone the promoterless *hspA* gene from *S. vulcanus* and *tac* promoter fused with the kanamycin resistance gene, respectively. B: Immunoblot analysis of crude soluble (indicated by Sup) and insoluble (indicated by Ppt) proteins extracted from the ECT and ECT16-1 cells grown at 30°C. Soluble and insoluble proteins extracted from a 10-μl aliquot of concentrated cultures whose absorbance at 730 nm was 2.4 were separated in each lane by SDS–PAGE and electroblotted onto a PVDF membrane. HspA, DnaK, and GroEL, were specifically detected using polyclonal antibodies raised against each protein from *S. vulcanus*.

coccus sp. PCC 7942, the gene was cloned downstream of the *tac* promoter. The shuttle vector pECAN8 [24] was used to introduce the recombinant DNA into *Synechococcus* sp. PCC 7942 (Fig. 1A). The HspA protein was overexpressed under the control of the *tac* promoter both in *E. coli* strain JM109 (data not shown) and *Synechococcus* sp. PCC 7942 (Fig. 1B). We designated the HspA-expressing cyanobacterial strain as ECT16-1, and the reference cell as ECT. Coomassie brilliant blue-stained protein profiles after SDS–PAGE showed a large accumulation of a 16-kDa protein in the soluble and insoluble fractions of the ECT16-1 cells which were grown at 30°C (data not shown). Anti-HspA from *S. vulcanus* cross-reacted with the 16-kDa protein in the soluble and insoluble fractions from ECT16-1 cells, but did not with proteins from cells of the reference strain (Fig. 1B). The HspA protein did not increase further upon heat shock at 45°C for 1 h (data not shown).

DnaK and GroEL have been shown to play a role in thermotolerance [1,2]. We tested whether the constitutive expression of HspA affects expression of these Hsps by immunoblot analysis following SDS–PAGE. As shown in Fig. 1B (lanes designated as 'Sup'), the level of DnaK in the ECT16-1 cells was similar to that in the ECT cells. Likewise, expression levels of GroEL were similar in both strains (Fig. 1B, lanes designated as 'Sup'). In Fig. 1B, quantitation was based on cell number. Similar experiments performed on a protein basis gave essentially the same results (data not shown). Interestingly, ~40% of the total cellular HspA was localized in insoluble fractions of the cell extracts (Fig. 1B; lanes designated as 'Ppt'), although DnaK and GroEL were present almost exclusively in soluble fractions.

Since a heat shock is not necessary to produce the HspA protein in this system and the constitutive expression of HspA does not affect expression of other major Hsps, the function of HspA can be studied without the induction of the other Hsps. Thus, the phenotype observed under stress conditions is a consequence of HspA expression. We therefore investigated whether *S. vulcanus* HspA could confer thermotolerance when expressed in *Synechococcus* sp. PCC 7942 cells.

3.2. Non-toxic constitutive expression of HspA protected the cell from thermal killing

The growth patterns of *E. coli* expressing a non-expressing HspA were identical, suggesting that overexpressed HspA exerted little if any effect on the normal growth of *E. coli* at 30, 37, 42 or 47°C (data not shown). Similarly, no difference in growth was observed between *Synechococcus* sp. PCC 7942 strains with and without HspA expression at 20 or 30°C (data not shown). Thus, overexpression of HspA was nontoxic to both *E. coli* and cyanobacteria.

Exponentially growing cells of ECT and ECT16-1 strains were heat-treated at 48°C for 1 h. On returning to 30°C, cells that survived resumed division at a normal rate after a lag period. There was a significant difference in recovery between the ECT and ECT16-1 cells at 30°C. The ECT16-1 cells showed a much shorter lag period (ca. 20 h) than the ECT cells (ca. 40 h). 40 h after returning to 30 from 48°C, the absorbance at 730 nm, an indication of cell number, was three

Table 1
Survival rate at 50°C of the ECT and ECT16-1 strains after a direct shift to 50°C either in the light (30 μE/m^2/s) or in the dark

Strain	Time (min) after a shift to 50°C		
	0	15	60
(A) Heat shock in the light			
ECT	100% (338 360 ± 94 518)	2.27 ± 0.93%	(2.03 ± 1.76) × 10^{-3}%
ECT16-1	100% (346 042 ± 97 631)	28.0 ± 6.1%	(13.9 ± 4.6) × 10^{-3}%
(B) Heat shock in the dark			
ECT	100% (336 426 ± 121 914)	32.0 ± 15.5%	
ECT16-1	100% (233 986 ± 47 861)	46.6 ± 14.9%	

In all the treatments shown, the number of cells present before the shift to 50°C was taken as 100%. The actual number of cells in a 10-μl aliquot of a non-diluted suspension before the heat-treatment is shown in parentheses. Each value shows the mean and standard deviation of four independent experiments.

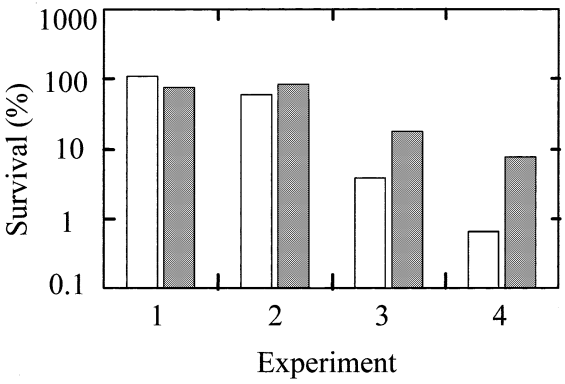

Fig. 2. The acquired thermotolerance of the ECT (open bars) and ECT16-1 (shaded bars) strains in different experiments. In each experiment cultures of ECT and ECT16-1 strains grown at 30°C were shifted together to a 50°C water bath for 60 min after a pretreatment at 45°C for 60 min. Survival rate (%) was expressed by taking the number of cells present before the shift to 50°C as 100%.

times higher in the overexpressed cells as compared with non-expressed cells.

We quantitatively measured cell survival after a high-temperature treatment by taking small aliquots of cell suspension during thermotolerance assays and spotting them on BG-11 agar plates at various dilutions. After the direct shift from 30 to 50°C for 15 min, 2.3% of the ECT cells survived, whereas 28% of the initial ECT16-1 cells were still viable (Table 1A). When cells were incubated at 50°C for 60 min, the survival rates for ECT and ECT16-1 were 2.0×10^{-3}% and 14×10^{-3}%, respectively (Table 1A). Thus, HspA increased the viability at 50°C. In order to examine the effect of HspA on acquired or induced thermotolerance, a similar assay was conducted after a pre-heat treatment of cells at 45°C for 1 h. We think this pretreatment is suitable for the development of thermotolerance in *Synechococcus* sp. PCC 7942 since this is the optimum temperature for the accumulation of GroEL in this cyanobacterium (data not shown) and the pretreatment did not kill the cells (data not shown). The adapted cells were challenged with a lethal temperature treatment at 50°C for 60 min. The mild heat pretreatment resulted in a 1000–10 000-fold increase in the survival rate at 50°C for 1 h in both ECT and ECT16-1 cells (compare results in Table 1A and Fig. 2). Although the survival rate varied between experiments, ECT16-1 tended to show a greater thermotolerance than ECT (Fig. 2). The level of the basal thermotolerance of the ECT16-1 strain at 50°C for 60 min was two to three orders of magnitude lower than the acquired thermotolerance of the pre-conditioned ECT cells. Thus, the expression of HspA alone is not sufficient to confer full protection to the cyanobacteria, although it clearly contributes to the acquired thermotolerance to some extent (Table 1A). When heat shock at 50°C for 15 min was performed in the dark, the survival rate for the ECT cells (32%) increased dramatically, approaching a similar level to that for ECT16-1 (28%) after the heat shock in the light (Table 1). The presence of HspA resulted in a 50% increase in the survival. Clearly, the protective effect of the HspA protein was more evident when the heat shock was given in the light. It appears that the adverse effect of light on viability during heat stress could be eliminated by the expression of a small Hsp.

3.3. *HspA plays a protective role against heat-induced damage to the photosynthetic apparatus*

Oxygen evolution rates were measured from the ECT and ECT16-1 strains after 15 min of exposure to 50°C. After the heat shock in the light, the ECT cells showed a complete inhibition of whole-chain electron transport (H_2O to $NADP^+$; Table 2A). Likewise, photosystem II (PS II) activity measured as a reduction of benzoquinone and ferricyanide using H_2O as electron donor was not detectable after the heat shock (Table 2A). In contrast to ECT, the ECT16-1 cells retained more than 30% of the oxygen evolution rates of whole-chain electron transport and PS II compared to before the heat shock (Table 2A).

We incubated the cultures at 50°C for 15 min in darkness in order to examine the effect of temperature specifically. In ECT cells, photosynthetic electron transport was completely inactivated by the treatment (Table 2B). The oxygen evolution rates in ECT16-1 for whole-chain electron transport and PS II were 22 and 8.5%, respectively, of the non-heat shock rate (Table 2B). The PS II activity was inactivated to approach the similar value for the whole-chain electron transport activity. The inactivation of PS II in the ECT16-1 strain was greater in the dark than in the light. The inactivation of photosynthetic electron transport activity in ECT and ECT16-1 cells was not reversible within 5 h of observation (data not shown).

We noticed that heat shock at 50°C for 15 min led to a change in the cell coloration of ECT. Thus, we measured pigment levels of the ECT and ECT16-1 cells. As shown in Fig. 3A, ECT showed a major decrease at 625 nm, which is associated with phycocyanin. On the contrary, there is almost no change in ECT16-1 (Fig. 3A). Chlorophyll content per cell did not change significantly in either ECT or ECT16-1 before

Table 2
Oxygen evolution rate (μmol O_2/ml/A_{730}/h) of the ECT and ECT16-1 strains after a direct shift to 50°C for 15 min either in the light (40 μE/m^2/s) (A) or in the dark (B)

Strain	30°C		50°C	
	Whole-chain electron transport	PS II electron transport	Whole-chain electron transport	PS II electron transport
(A) Heat shock in the light				
ECT	1.41 ± 0.09	4.85 ± 0.15	ND	ND
ECT16-1	1.75 ± 0.13	5.51 ± 0.29	0.617 ± 0.076	1.80 ± 0.11
(B) Heat shock in the dark				
ECT	1.87 ± 0.03	5.04 ± 0.26	ND	ND
ECT16-1	1.56 ± 0.12	5.65 ± 0.27	0.347 ± 0.013	0.483 ± 0.066

Oxygen evolution was measured in the presence of 10 mM $NaHCO_3$ (whole-chain electron transport) or in the presence of 1 mM 1,4-benzoquinone and 1 mM $K_3Fe(CN)_6$ (PS II electron transport) either before (designated by 30°C) or after (designated by 50°C) the shift to 50°C. Average values and standard deviations from at least three measurements are given. ND, not detectable.

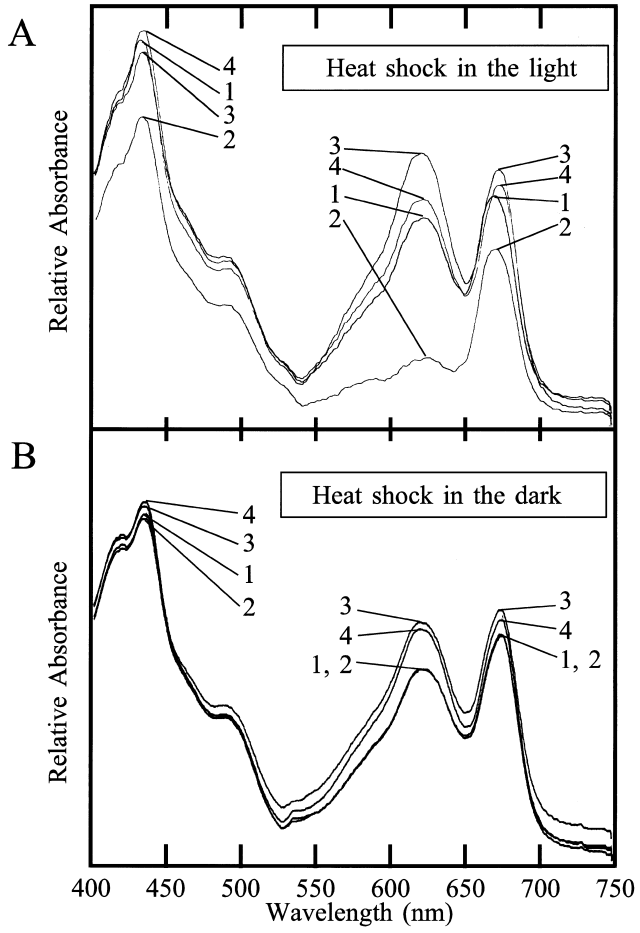

Fig. 3. Absorption spectra of the ECT (traces 1 and 2) and ECT16-1 (traces 3 and 4) cells grown at 30°C either before (traces 1 and 3) or after (traces 2 and 4) the shift to 50°C for 15 min in the light (40 μE/m^2/s) (A) or in darkness (B).

and after the heat shock (Fig. 3A and data not shown). This loss in the absorption due to phycocyanin was not reversible, at least within 24 h. When similar experiments were performed in the dark, there was no change in the absorption spectra of both ECT and ECT16-1 cells before and after the heat shock (Fig. 3B).

4. Discussion

One of the genetic approaches to demonstrate a specific contribution of a Hsp in thermotolerance is the selection, construction, and phenotype analysis of mutants and transgenic organisms in which expression of a specific Hsp is either completely inactivated, partially repressed, or over-expressed. Until now, three Hsp genes in cyanobacteria, *clpB* [27], *htpG* [28], and *hsp16.6* [19] have been inactivated. However, there has been no report on the constitutive (over)production of any Hsps in cyanobacteria. In the present study, we succeeded in the construction of a mutant strain which constitutively expresses HspA, a small Hsp homologue from *S. vulcanus*. Unlike DnaK and GroEL, small Hsps in cyanobacteria such as *S. vulcanus*, *Synechocystis* sp. PCC 6803, and *Synechococcus* sp. PCC 7942 appear to be expressed only under stress conditions [18]. Thus, we rationalized that the effect of HspA expression on the survival or growth rate can be evaluated without the influence of endogenous small Hsp in a cyanobacterium when cells grown under non-stressed conditions are directly exposed to a lethal temperature. The HspA expressing strain showed faster recovery and higher survival rate when cells were directly shifted to a lethal temperature (50°C) for 15 or 60 min in the light, indicating a significant contribution of a single cyanobacterial small Hsp in the acquired thermotolerance. However, the thermotolerance acquired by the expression of the HspA protein could not compare with that acquired by a mild heat-pretreatment.

We showed that HspA can protect PS II from heat-inactivation. Lee et al. [19] found that the *Synechocystis* mutant lacking Hsp16.6 displayed a greater reduction in oxygen evolution rates than the wild type after heat-shock treatment [19]. However, there was no evidence as to which system, PS I or PS II, is specifically impaired since they only measured whole-chain electron transport. The PS II complex, and particularly the oxygen-evolving machinery, is recognized to be the most heat-sensitive of the chloroplast thylakoid–membrane protein complexes involved in photosynthetic electron transport and ATP synthesis [29,30]. Recently, evidence was obtained from a higher plant-chloroplast reconstituted system on the involvement of chloroplast small Hsp in protection of thermolabile PS II during heat stress [31]. Functional disruption of small Hsp using anti-small Hsp antibodies or addition of purified small Hsp to thylakoid membranes indicated that the Hsp completely accounted for acquired thermotolerance of PS II in pre-heat-stressed plants. Our present results with an in vivo system appear to support their data.

There is some evidence of a physical interaction between small Hsp and thylakoids. The nuclear-coded small Hsp (Hsp22), found in chloroplasts of pea and the green alga *Chlamydomonas reinhardtii*, associated with the thylakoid membranes when the precursor to Hsp22 had been transported into intact chloroplasts from heat-shocked plants [32–34]. Hsp22 was found mostly in the grana-membrane subfraction where PS II and the associated light-harvesting chlorophyll *a/b* complexes are located. The vast majority of newly synthesized Hsp16.6 from *Synechocystis* sp. PCC 6803 was also associated with thylakoids [35]. Supporting their observation, we showed that HspA was localized in the insoluble fraction of the cell extracts as well as the soluble fraction under normal conditions. Upon heat shock, the proportion of HspA that fractionated in the insoluble fraction increased further (data not shown). Collectively, these results suggest that HspA may form a stable complex with denatured proteins in thylakoid membrane. Experiments such as immunogold localization of HspA using transmission electron microscopy are in progress in order to identify the actual functional role of HspA.

We showed that HspA can protect the light absorbing ability of phycocyanin. Phycocyanin is a constituent of phycobilisomes, large multiprotein complexes that are located on the cytoplasmic side of the thylakoid membrane [36]. Phycobilisomes serve as the primary light-harvesting antennae for photosynthesis in cyanobacteria and red algae. As far as we know, this is the first indication that a small Hsp is involved in the thermostability of a light-harvesting complex of photosynthetic prokaryotes. The loss of absorption by phycocyanin took place in the light, but not in darkness under our experimental conditions. Previously, it was shown that exposure of

cyanobacterial cells to high intensity white light (6700 μE/m^2/s) at 58.5°C for 15 min led to losses of both chlorophyll *a* and phycocyanin absorption [37]. Their experiments also showed that phycocyanin is more susceptible to photobleaching at high temperatures than chlorophyll *a*.

Chloroplast Hsps in eukaryotes have been proposed to be involved in the protection of PS II against damage by light (i.e. photoinhibition) during heat shock [34]. Although the light intensity used in the present study (30 μE/m^2/s) was low, photoinhibition of photosynthetic electron transport might take place at lower-light intensities during heat shock than under normal conditions [34]. However, the inhibition of PS II activity observed in the present study took place in the dark as well as in the light (Table 2). Thus, we conclude that HspA protects PS II from thermal inactivation under our experimental conditions. Although PS II in the reference strain ECT was completely inactivated in the dark and the inactivation was not readily recovered within several hours, the survival rate in the dark was significantly higher than that in the light (Table 1). Thus, there is no correlation between the cellular death and PS II inactivation. Cellular death of cyanobacteria following exposure to heat stress appeared to be more closely correlated with damage to a light-harvesting pigment phycocyanin than to PS II.

In conclusion, the results we presented show that small Hsp plays a particularly important role when cyanobacteria are stressed by heat and light together, which is likely to be a common condition for many photoautotrophs such as cyanobacteria in their natural habitat.

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