A small heat shock protein confers stress tolerance and stabilizes thylakoid membrane proteins in cyanobacteria under oxidative stress

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Summary

Small heat shock proteins are molecular chaperones that bind and prevent aggregation of nonnative proteins. They also associate with membranes. In this study, we show that the small heat shock protein HspA plays a protective role under oxidative stress in the cyanobacterium *Synechococcus elongatus* strain ECT16-1, which constitutively expresses HspA. Compared with the reference strain ECT, ECT16-1 showed much better growth and viability in the presence of hydrogen peroxide. Under the peroxide stress, pigments in thylakoid membrane, chlorophyll, carotenoids, and phycocyanins, were continuously reduced in ECT, but in ECT16-1 they decreased only during the first 24 h of stress; thereafter no further reduction was observed. For comparison, we analyzed a wild type and an *hspA* deletion strain from *Synechocystis* sp. PCC 6803 and found that lack of *hspA* significantly affected the viability of the cell and the pigment content in the presence of methyl viologen, suggesting that HspA stabilizes membrane proteins such as the photosystems and phycobilisomes from oxidative damage. *In vitro* pull down assays showed a direct interaction of HspA with components of phycobilisomes. These results show that HspA and, small heat shock proteins in general play an important role in the acclimation to oxidative stress in cyanobacteria.

Key words: Cyanobacterium, oxidative stress, photosystem, phycobilisome, phycocyanin, small heat shock protein

Introduction

Cyanobacteria are photosynthetic prokaryotes that have been used as model organisms for detailed molecular analyses of oxygenic photosynthesis. Cyanobacterial light-harvesting antenna systems consist of phycobiliproteins that form a supramolecular assembly, the phycobilisome (PBS), located on the cytoplasmic surface of the thylakoid membrane (Sidler 1994; MacColl 1998; Adir 2005). Light energy is absorbed by PBS, then transferred to the photosystems. Phycobiliproteins such as phycocyanins are colored, water-soluble proteins that contain covalently linked, open-chain tetrapyrroles known as phycobilins. In addition, PBS also contains smaller amounts of linker polypeptides, which are vital for proper assembly and functional organization of the complex.

The light energy absorbed by PBS is converted into chemical energy by photosystem I and II (PSI and PSII). PSI and PSII are membrane-bound multisubunit protein complexes that cooperate to transfer electrons from water to $NADP^+$ (Nelson and Yocum 2006). PSI and PSII consist of a number of extrinsic and integral membrane proteins. PSII carries out the light-driven oxidation of water and reduction of plastoquinone, while PSI carries out the light-driven oxidation of plastocyanin/cytochrome c_6 and reduction of ferredoxin/flavodoxin.

Cyanobacteria in their natural environments may encounter oxidative stress caused by high temperature, high light intensity, and other physicochemical changes. PSI and PSII are the major generation sites of reactive oxygen species (ROS). PSI reduces molecular oxygen to superoxide anion (Asada 2006), which is converted by superoxide dismutase to hydrogen peroxide. In PSII, oxygen of the ground (triplet) state ($^3O_2$) is excited to singlet state ($^1O_2$) by the reaction center chlorophyll of triplet excited state (Telfer et al. 1994; Asada 2006). When either the intensity of photosynthetically active radiation is too high or the cell's capacity to use absorbed light energy is lowered by environmental stresses, excess photons promote the production of ROS and cause the reduction of the efficiency and/or maximum rate of photosynthesis termed photoinhibition. Under such conditions, PSI and PSII are damaged (Hihara and Sonoike 2001; Murata et al. 2007). Recently, we have reported that PBSs are also photodamaged under heat stress (Nakamoto and Honma 2006).

Small Hsps are ubiquitous in the biological world (Kappé et al. 2002; Nakamoto and Vigh 2007). *In vitro*, small Hsps bind and stabilize non-native proteins to prevent their aggregation during stress (van Montfort et al. 2001; Haslbeck et al. 2005; Nakamoto and Vigh 2007). While small Hsp-stabilized proteins alone do not refold spontaneously, they are delivered to a multichaperone network where they refold in an ATP-dependent manner (Veinger et al. 1998). Two well characterized bacterial small Hsps are the *E. coli* inclusion body-associated proteins IbpA and IbpB (Allen et al. 1992). The *ibpA*-, *ibpB*- and *ibpAB*-overexpressing strains were found to be resistant to superoxide stress (Kitagawa et al. 2000). However, the *ibpAB*-disrupted strain failed to be more sensitive to these stresses than the wild-type strain (Kitagawa et al. 2000). Recently, Matuszewska et al. (2008) demonstrated that IbpA/B are involved in protection against copper induced oxidative stress. Although nothing is known about the *in vivo* role of small Hsps under oxidative stress in cyanobacteria, DNA microarray analysis showed that a gene encoding HspA, a small Hsp, is the gene most strongly induced by peroxide among genes encoding major Hsps in the cyanobacterium *Synechocystis* sp. strain PCC 6803 (Li et al. 2004). The high induction of this small Hsp in the cyanobacterium would seem to indicate that it plays a role under oxidative stress. In addition to *hspA*, other chaperone genes including *groESL1*, *groEL2*, *htpG*, and *dnaJ* paralogs are induced by peroxide stress (Li et al. 2004). Thus, HspA may collaborate with other chaperones in order to protect cells against oxidative stress.

In order to understand the role of small Hsps under oxidative stress *in vivo* in cyanobacteria, we characterized the phenotype of the *Synechococcus elongatus* strain ECT16-1 harboring an *hspA* expression vector with that of *Synechococcus elongatus* strain ECT, the reference strain, which harbors the vector without the *hspA* gene. ECT16-1 constitutively expresses HspA without affecting expression of other major Hsps such as GroEL and DnaK under normal growth conditions (Nakamoto et al. 2000). The HspA is localized in thylakoid membranes as well as cytosol (Nitta et al. 2005). In the present study, we also used an *hspA* deletion mutant of *Synechocystis* sp. strain PCC 6803 (Asadulghani et al. 2004) in order to show the importance of HspA under oxidative stress in cyanobacteria other than *Synechococcus*. The *hspA* mutant shows elevated levels of the

groESL1, *groEL2*, and *htpG* mRNAs as compared with the wild type (Asadulghani et al. 2004).

Our present results clearly showed the importance of small Hsp under oxidative stress. Expression of HspA enhanced tolerance against oxidative stress in *Synechococcus*, while a deletion in the *hspA* gene led to high sensitivity to oxidative stress in *Synechocystis*. We identified PBS and PSII/PSI, the major thylakoid membrane proteins, as apparent targets for small Hsp in cyanobacteria, indicating that small Hsp plays a role in membrane quality control.

Methods and methods

Strains

To create *Synechococcus elongatus* strain ECT16-1 (ECT16-1), a shuttle vector harboring the *hspA* gene encoding a small Hsp from the thermophilic cyanobacterium *Synechococcus vulcanus* was introduced into *Synechococcus elongatus* strain PCC 7942 for constitutive expression of the HspA protein (Nakamoto et al. 2000). In the vector, the *hspA* gene was placed under the control of the *tac* promoter. In *Synechococcus elongatus* strain ECT (ECT), a reference strain, the shuttle vector without the *hspA* gene was introduced in the same manner as in the ECT16-1 strain for a control. To create a kanamycin-resistant *hspA* mutant of *Synechocystis* sp. strain PCC 6803, the entire *hspA* coding region was deleted (Asadulghani et al. 2004).

Oxidative treatment

Cells were cultured at 30ºC in BG-11 inorganic liquid medium (Nakamoto et al. 2000; Asadulghani et al. 2004), to early exponential phase (when the apparent absorbance at 730 nm of the culture was between 0.2 and 0.3) and hydrogen peroxide (0.8 to 1.0 mM) or methyl viologen (1 to 4 μM) was added. The cultures were incubated under the standard growth conditions (continuous air bubbling, 30ºC, 30 $\mu E/m^2/s^{-1}$ light intensity) throughout the treatment.

Measurement of growth and whole cell absorbance

The growth rate of the cultures was determined by measuring apparent absorbance at 730 nm of diluted cultures using a Shimadzu UV-1200 double beam spectrophotometer. Whole cell absorbance was measured at room temperature with a Hitachi 557 double wavelength double beam spectrophotometer.

Viability assay

Cells were grown to early exponential phase as described above and then hydrogen peroxide or methyl viologen was added. Every 24 h an aliquot culture was sampled to

determine cell survival as described previously (Nakamoto et al. 2000). At least three independent replicate experiments with different cultures were carried out for all assays.

Protein analysis

Cells were disrupted by a method described previously (Roy et al. 1999). The cell extract was centrifuged at 16,000 g for 30 min at 4°C. The supernatant fraction (40 μg of protein), which was dissolved in 10 μl of the SDS-sample buffer containing 0.06 M Tris/HCl (pH 6.8), 0.06 M dithiothreitol, 6% sucrose, 2% sodium dodecyl sulfate (SDS) and 0.03% bromophenol blue), was heated for 3 min and then electrophoresed in a 15% SDS–polyacrylamide gel as described previously (Roy et al. 1999). Immunoblot analysis using anti-*Synechococcus vulcanus* HspA, anti- *Synechococcus vulcanus* DnaK, and anti-*Synechococcus vulcanus* GroEL polyclonal antibodies as probes was performed as described previously (Roy et al. 1999).

For whole cell protein analysis, cells from cultures (2 ml), whose apparent absorbance at 730 nm was 0.1, were harvested by centrifugation and 20 μl of the above SDS-sample buffer was added. The cell suspension was mixed well, and then incubated at 100ºC for 5 min. The samples were electrophoresed as described above.

In-gel detection of phycocyanin fluorescence

The method by Raps (Raps 1990) was modified. After polypeptides were separated by SDS-PAGE, 1 ml of 100 mM $ZnSO_4$ was spread over the gel to intensify phycocyanin fluorescence. The fluorescence, which was excited by 532 nm illumination and passed through a 640 nm band path filter, was detected using Molecular Imager FX (Bio-Rad).

Plasmid construction and overexpression of HspA in *E. coli*

The gene encoding HspA was PCR amplified from *Synechococcus* sp. PCC 7942 chromosomal DNA with two oligonucleotide primers, HspA-F(5-CATATGGCACTCGTTCGATTC-3) and HspA-R(5-CTCGAGTCGCTCGCAAGCTTCAG-3). The amplified fragment was cloned into pT7Blue Vector (Invitrogen) according to the manufacturer's instructions. This

plasmid was digested with *Nde*I and *Xho*I and the insert was subsequently cloned into the pET21a expression vector (Invitrogen), previously digested with the same restriction enzymes. The vector pET21a-HspA carried a His6 tag which was fused to the C terminus of the *hspA* gene product. The constructed plasmid was transformed into *E.coli* BL21(DE3). The cells were grown to apparent absorption 0.6 at 540 nm in LB medium containing 100 μg/ml ampicillin at 37°C, and then IPTG was added to a concentration of 1 mM. After a further 3 h at 37°C, the cells were harvested by centrifugation and stored at -80°C.

Purification of the His-tagged HspA protein.

All the following procedures were carried out at 4^{o}C unless stated otherwise. Cells were suspended in 25 mM Tris (pH 7.5), 1 mM EDTA, 2 mM DTT, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride and 1 mM caproic acid, disrupted by sonication and cell debris was removed by centrifugation at 16,000 g for 30 min. The supernatant was fractionated using solid ammonium sulfate. The proteins which precipitated between 0 and 60% saturation were suspended in 25 mM Tris (pH 7.5) containing 1 mM EDTA, dialyzed overnight against the same buffer, and loaded onto a column of DEAE/Toyopearl 650S (Tosoh) equilibrated with 20 mM Tris (pH 8.0), 1 mM EDTA. The column was washed with 20 mM Tris (pH 8.0) containing 1 mM EDTA. Proteins were eluted with 50 ml of 0-500 mM NaCl linear gradient in 20 mM Tris (pH 8.0), 1 mM EDTA. Fractions containing HspA were pooled and dialysed overnight against 5 mM Na-phosphate (pH 7.5), then loaded onto a column of hydroxylapatite HP40-100 (Asahi Optical) equilibrated with 5 mM Na-phosphate (pH 7.5). The column was washed with the same buffer, and then proteins were eluted with 60 ml of 0-400 mM Na-phosphate (pH 7.5) linear gradient. The HspA elution started at approximately 50 mM Na-phosphate. Fractions containing HspA were pooled and dialysed overnight against an appropriate buffer.

Bleaching of PBS *in vitro*

PBS from exponentially growing cells of 1 liter culture was isolated by a previously described procedure (Yamanaka et al. 1978; Nakamoto and Honma 2006). Isolated PBS, purified HspA, bovine α-crystallin, or lysozyme was dialyzed against 0.75 M

NaK-phosphate (pH 8.0). A mixture (200 μl) of these dialyzed proteins was incubated in the same buffer containing 32 or 160 mM hydrogen peroxide at 30°C for 9 h or 18 h, and then chilled on ice. The PBS mixture was complemented to 1 ml with the NaK-phosphate, and absorption at 620 nm was measured by a Shimadzu UV-1200 double beam spectrophotometer.

Pull down assays

Each solution containing PBS or His-HspA was dialysed against 0.75 M NaK-phosphate (pH 8.0). A mixture (700 μl) containing PBS (331 μg/ml) and/or His-HspA (132 μg/ml, 6.8 μM) was incubated in the same buffer containing 160 mM hydrogen peroxide at 30°C for 9 h. After centrifugation at 16,000 g, the supernatant was loaded onto Ni-chelating resins (Profinity IMAC Resins, Bio-Rad) and incubated at 4°C for 2 h with gentle rotary stirring. After washing the resins with 20 mM NaK-phosphate (pH 8.0) containing 1% Triton X-100 4 times, the above SDS-sample buffer was added to the resins. The sample was boiled for 5 min and resolved by electrophoresis on a 15% SDS-polyacrylamide gel. The gel was stained by CBB R-250 and phycocyanin fluorescence was detected as described above.

Results

Effect of hydrogen peroxide on the *Synechococcus elongatus* strains ECT and ECT16-1.

In order to test whether small Hsp plays a role in the oxidative stress response, we compared the effect of hydrogen peroxide on the ECT16-1 strain harboring a *Synechococcus vulcanus hspA* expression vector with that on the ECT strain, the reference strain, which harbors the vector without the *hspA* gene.

Growth curves for ECT and ECT16-1 in the presence of 1 mM hydrogen peroxide at 30ºC under a light intensity of 30 μE m^{-2} s^{-1} are shown in Fig. 1a and reveal that the ECT strain had a much lower growth rate than the ECT16-1 strain. Growth of ECT did not

resume within 72 h, but ECT16-1 grew continuously. These results suggest that HspA enhances cell survival and tolerance to peroxide stress. Thus, we measured cell viability.

When ECT and ECT16-1 cells were incubated for 24 h with hydrogen peroxide, 29% of ECT16-1 cells remained viable, whereas the viability of ECT cells decreased dramatically to 3% (Fig. 1b). The viability of both strains increased gradually after 24 h incubation. After a 72 h incubation period the percentage of viability was 66% in ECT 16-1, while that of ECT was only 10%. The increase in viability after more than 24 h may be due to the decomposition of hydrogen peroxide in the cultures during the incubation period.

The ECT culture completely bleached during the 72-h incubation period. We determined the cellular contents of photosynthetic pigments by measuring whole cell absorbance. The light absorption at 460 nm, 620 nm, and 675 nm is associated with carotenoids, phycocyanins, and chlorophyll a, respectively. ECT showed a major decrease in the absorbance by carotenoids, phycocyanins, and chlorophyll a (Fig. 1c). The reduction of carotenoids and chlorophyll content was more pronounced than that of phycocyanins. Among the pigments, carotenoids decreased most quickly after the initiation of the stress. Although there was a small decrease in these absorbances in ECT16-1 cells within 24 h after the addition of hydrogen peroxide, no further decrease occurred in ECT16-1 after that (Fig. 1d).

We examined whether constitutive expression of HspA does not affect expression of other major Hsps during the oxidative stress treatment. Enhanced expression of other chaperones may also contribute to the increase in tolerance to the stress. Immunoblot analysis revealed that levels of GroEL and DnaK remained unaffected during incubation of ECT and ECT16-1 cells with hydrogen peroxide (Fig. 2). The accumulation of HspA in ECT16-1 increased gradually.

We examined effects of lower concentrations of hydrogen peroxide on growth, survival and pigment levels of the two strains (supplementary Figs. 1 and 2). When the concentration was lowed below 1 mM, decrease in survival rate and the pigment levels was relieved in ECT as compared with that in the presence of 1 mM hydrogen peroxide. Compared with ECT, ECT16-1 showed better viability in the presence of 0.8 or 0.9 mM

hydrogen peroxide, although its growth was greatly inhibited. No significant decrease in the pigments was observed in ECT16-1.

Phycocyanin analysis

The decrease in absorbance at 620 nm (Fig. 1c) indicates a decrease in phycocyanin content in ECT. Phycocyanins are major components of PBSs (Yamanaka et al. 1978), which could be easily detected in a CBB-stained gel after SDS-PAGE. Both phycocyanin α and β subunits were greatly reduced in the stressed ECT cell, but in the ECT16-1 strain the level of these subunits was constant until the end of the 72 h stress period, after a moderate reduction during the first 24 h period (Fig. 3a). Three non-pigmented linker polypeptides of 33-, 30-, and 27-kDa were more rapidly lost in ECT than in ECT16-1, and became absent after 72-h incubation with hydrogen peroxide in ECT cells. The much greater decrease in phycocyanin content in ECT than in ECT16-1 was confirmed by specific fluorometric detection of phycocyanins (Fig. 3b). Fig. 3b also showed that the allophycocyanin β band decreased rapidly in ECT.

Direct interaction of HspA with phycocyanin

In order to test whether HspA interacts with phycocyanins directly to protect them in the presence of hydrogen peroxide, we examined its effect on the hydrogen peroxide-dependent bleaching of phycocyanins *in vitro*. For these *in vitro* experiments, PBS was isolated from *Synechococcus elongatus* strain PCC 7942 which is the background strain for the construction of ECT and ECT16-1. His-tagged HspA of the same cyanobacterium was overexpressed in *E. coli* and purified. We tested various concentrations of hydrogen peroxide which can cause bleaching of phycocyanins of PBS at 30^{o}C, the growth temperature for ECT and ECT16-1. We found that 32 mM and 160 mM hydrogen peroxide bleached phycocyanins *in vitro*, resulting in 20% and 85% loss, respectively, of absorption at 620 nm during 24 h of incubation at 30ºC (data not shown). In the following *in vitro* experiments, we employed 160 mM hydrogen peroxide to reconstruct the phycocyanin bleaching that takes place to a reasonable extent within a reasonable time. We examined effect of lysozyme or HspA on the bleaching. Both

proteins (up to 90 μg per 30 μg PBS) did not change the phycocyanin absorbance at all in the absence of hydrogen peroxide. Adding an amount of HspA (30 μg) equal in mass to the mass of PBS suppressed the bleaching in the presence of hydrogen peroxide, whereas the same amount of lysozyme did not (Fig. 4a). In terms of molar ratio in the reaction mixture, lysozyme per phycocyanins (10 μM/6.1 μM = 1.7) was higher than HspA/phycocyanins (7.7 μM/6.1 μM = 1.3). Phycocyanins account for about 75% of the total PBS proteins (Yamanaka et al. 1978). Thus, it is possible to calculate the molar concentration of phycocyanins based on the mass of PBS. Lysozyme has been often used as a control protein in chaperone activity measurements. Bovine lens α-crystallin (7.5 μM), another small Hsp, also showed a similar protective effect (Fig. 4a), indicating that small Hsps in general offer protection against bleaching to some extent. Among various small Hsps, α-crystallins have been most intensively characterized (Nakamoto and Vigh, 2007).

In order to confirm the direct interaction between HspA and the components of PBS, we performed pull down assays (Fig. 4b). Phycocyanins as well as linker polypeptides were pulled down by His-tagged HspA when both HspA and PBS were incubated at 30^{o}C for 9 h in the presence of 160 mM hydrogen peroxide. No phycocyanins were pulled down when they were incubated in the presence of hydrogen peroxide on ice or at 30^{o}C in the absence of hydrogen peroxide (data not shown). These results indicate that HspA can interact directly with oxidatively damaged PBS, and thereby stabilize it to some extent under the present *in vitro* conditions.

Effect of methyl viologen on *Synechococcus elongatus* **strain ECT16-1 and an** *hspA***-deletion mutant of** *Synechocystis***.**

In order to collect more information about the role of HspA in the protection of cells under oxidative stress, we compared the effect of methyl viologen on ECT16-1 with that on ECT. Hydrogen peroxide added may be decomposed in the cyanobacterial cultures. In order to ascertain that HspA does play a role under oxidative stress, we decided to use methyl viologen in order to produce active oxygen in a cell.

Methyl viologen accepts electrons from the photosynthetic electron transport system, generating active oxygen species (e.g., superoxide anion, hydrogen peroxide, hydroxyl radical) which are highly reactive to cellular macromolecules (Bus and Gibson 1984). There was only a slight difference in either growth or viability of the two strains at 30ºC under a light intensity of 30 μE m^{-2} s^{-1} in the presence of 1 μM methyl viologen (Figs. 5a and 5b). Whole cell absorption spectra reveal that cellular contents of all these pigments dropped transiently after 24 h of incubation and then recovered again in ECT (Fig. 5c), while they remained fairly constant in ECT16-1 (Fig. 5d). It is probable that the transient reduction or bleaching of the photosynthetic pigments was caused by active oxygen produced by methyl viologen, and that pigment levels were recovered after acclimation of cells to the stress. The result suggests that expression of HspA suppresses reduction of cellular levels of phycocyanins and chlorophylls due to the oxidative stress exerted by methyl viologen. We examined effects of higher concentrations of methyl viologen on growth and chlorophyll level of the two strains. 2 μM methyl viologen showed an inhibitory effect on growth of both strains. No or only slight growth of the two strains was observed in the presence of 3 μM methyl viologen. Under these conditions, we did not observe a beneficial effect of the HspA expression on growth. However, the reduction of chlorophyll, carotenoid and phycocyanin content that was observed in both strains at 3 μM methyl viologen was more pronounced in ECT than in ECT16-1. These results also indicate that HspA suppresses reduction of cellular levels of photosynthetic pigments due to the oxidative stress.

We have previously shown that HspA is greatly induced in the presence of methyl viologen in *Synechocystis* sp. strain PCC 6803 (Kojima et al. 2006), suggesting a role of HspA in the process of acclimation to this stress in *Synechocystis*. In order to elucidate that HspA plays a role under oxidative stress in cyanobacteria other than *Synechococcus*, we compared the effect of methyl viologen on an *hspA* deletion mutant of *Synechocystis* sp. strain PCC6803 (Asadulghani et al. 2004) with that on the wild type strain.

The lack of *hspA* in *Synechocystis* had a highly negative effect on growth and viability at 30ºC under a light intensity of 30 μE m^{-2} s^{-1} in the presence of 4 μM methyl viologen; see Figs. 6a and 6b for a comparison with the wild type. The mutation greatly reduced the

level of carotenoids, chlorophylls, and phycocyanins as shown by the absorption changes (Fig. 6d). The pigment levels kept dropping in the mutant, while they dropped transiently after 24 h of incubation in the wild type (Fig. 6c). No carotenoids were detected in the mutant after 72 h of incubation. The reduction of carotenoids and chlorophyll content was more pronounced than that of phycocyanins. Thus, the phycobilin to chlorophyll ratio increased during the 72-h incubation. These results with *Synechocystis* cells suggest that HspA plays an important role in conferring cellular tolerance against oxidative stress in *Synechocystis*. The decrease in absorbance at 620 nm (Fig. 6d) indicates a decrease in phycocyanin content in the *hspA* deletion mutant of *Synechocystis*. When detected in a CBB-stained gel after SDS-PAGE, allophycocyanin β decreased to a non-detectable level within the 48 h incubation (data not shown).

At 3 μM methyl viologen, both strains grew and there was no difference in their growth. However, chlorophyll on a per-cell basis in the *hspA* mutant decreased more than that in the wild type. These results are consistent with the results shown in Fig. 5. Thus, HspA has a protective effect on the pigment content under non-lethal oxidative stress conditions as well as lethal conditions.

Discussion

In the present study, we have shown that *Synechococcus* grows better and has a higher survival rate under peroxide stress when HspA was constitutively expressed in cells (Fig. 1), indicating a significant contribution of a single cyanobacterial small Hsp to the stress tolerance. In *Synechocystis*, the deletion of the *hspA* gene resulted in reduction of both growth and survival in the presence of methyl viologen (Fig. 6). As described in the introduction, expression of other molecular chaperones is enhanced under normal growth conditions in this mutant (Asadulghani, 2004). However, the enhanced expression does not seem to result in protection of cells under the oxidative stress (Fig. 6). Thus, small Hsp may play a global and unique role in conferring oxidative stress tolerance to cyanobacterial cells.

We also showed that the chlorophyll content was greatly decreased in the ECT strain and the *hspA* deletion mutant during the 72-h incubation of cells in the presence of methyl viologen or hydrogen peroxide (Figs. 1 and 6). It is known that PSII and PSI are highly susceptible to oxidative stress (Hihara and Sonoike 2001; Murata et al. 2007). The great reduction in the cellular level of chlorophylls must be due to the destruction of PSII and PSI since essentially all chlorophylls are bound by these complexes. Thus, PSII and/or PSI proteins are targets of oxidative damage. Expression of HspA resulted in suppression of the degradation under oxidative stress (Figs. 1 and 6).

Furthermore, constitutive expression of HspA protected PBS in the presence of methyl viologen or hydrogen peroxide (Figs. 1 and 6). The cellular absorbance at 620 nm decreased continuously during oxidative stress on the ECT strain and the *hspA* deletion mutant, indicating that expression of HspA resulted in suppression of the bleaching or degradation of phycocyanins. Protein analysis by SDS-PAGE showed that the absorbance decrease is due to the degradation of phycobilisomes (Fig. 3).

HspA suppressed the phycocyanin absorbance decrease in the presence of hydrogen peroxide by direct interaction with the component proteins of PBS (Fig. 4), indicating that PBSs are stabilized by the interaction with HspA. However, we observed only a 20% stability increase in the presence of HspA or α-crystallin. In the *in vitro* experiment, HspA equal in mass to the mass of PBS was present in a reaction mixture. In ECT16-1, HspA accumulated less than phycocyanins (data not shown). Thus, HspA appears to function much more efficiently *in vivo*. These results suggest that the binding of small Hsp to PBSs may not be sufficient to fully protect them under oxidative stress. Small Hsp can efficiently bind unfolded proteins to prevent their aggregation (Nakamoto and Vigh 2007). The denatured proteins bound to small Hsp must be delivered to the DnaK/DnaJ/GrpE system for them to refold (Veinger et al. 1998). Thus, the interaction between small Hsp and denatured proteins may be transient *in vivo*, and it functions like an enzyme. The interaction between small Hsp and the denatured proteins is not transient in the absence of the DnaK system, and this may be the reason why HspA appears to be inefficient in our *in vitro* experiments.

Our present study showed that allophycocyanin β separated by SDS-PAGE disappeared in ECT (Fig. 3b) and the *hspA* mutant (data not shown) during an early phase of the peroxide stress. Low temperature fluorescence emission measurement indicated that H_2O_2 treatment of *Synechocystis* cells for 10 min in darkness induces disassembly of the core of PBS and interruption of energy transfer from allophycocyanin to the terminal emitter (Liu et al. 2005). The disassembly may play a role to protect cells against the oxidative stress. The disassembled core of PBS may be more susceptible to proteases than that in the assembled PBS bound to thylakoid membrane.

There is some evidence of a physical interaction between small Hsps and thylakoid membrane/membrane proteins in cyanobacteria. The vast majority of newly synthesized Hsp16.6 (HspA) from *Synechocystis* sp. strain PCC 6803 is associated with thylakoids (Horváth et al. 1998). We have previously detected the HspA protein in thylakoids as well as in the cytosol by immunocytochemical studies (Nitta et al. 2005). Our results suggest that HspA may form a complex with thylakoid membrane proteins of PSI, PSII, and PBS in order to protect them from degradation during oxidative stress.

In the above discussion, we focused on direct effect of HspA on the photosystems and PBS. However, we cannot exclude that the observed effect is indirect. The previously well-documented, specific interaction of small Hsp with membrane lipids is likely an additional factor which ultimately leads to the stabilization of membranes during oxidative stress (Török et al., 2001). HspA (Hsp17) interacts preferentially with the liquid crystalline phase and resulted in an elevated physical order in lipid membranes. Interestingly, Török et al. (2001) also showed that HspA penetrates into the membrane hydrophobic core. Thus, HspA may interact with membrane-embedded proteins as well as proteins located on membrane surface. We showed that constitutive expression of HspA in *Synechococcus* stabilizes thylakoid membranes during heat stress and increases the thermal resistance of photosystem II (Nakamoto et al. 2000, Nitta et al. 2005), indicating that HspA is involved in quality control of membranes and membrane proteins.

In conclusion, our present results clearly prove that small Hsp is involved in oxidative stress management in cyanobacteria.

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Figure legends

Fig. 1. Cell growth (**a**), viability (**b**), and cellular absorption spectra of the *Synechococcus* ECT (**c**) and ECT16-1 (**d**) strains incubated in the presence of 1 mM hydrogen peroxide for 0, 24, 48, and 72 h. Diamonds and squares (**a** and **b**) represent ECT and ECT16-1, respectively. Cells were cultured at 30ºC to approximately 0.3 apparent absorbance at 730 nm, and then (at 0 h) hydrogen peroxide was added to a final concentration of 1 mM. Results in **a** and **b** are averages of three independent experiments; bars indicate standard deviations. **c** and **d** show typical curves obtained in one of three experiments.

Fig. 2. Immunoblot analysis of crude soluble proteins extracted from the ECT and ECT16-1 cells incubated in the presence of 1mM hydrogen peroxide for 24, 48, or 72 h. 40 μg of proteins 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 *Synechococcus vulcanus*.

Fig. 3. Separation of proteins of whole cell by SDS-PAGE (**a**) and detection of phycocyanin fluorescence by Molecular Imager FX (**b**). In **a** and **b**, samples each containing cells of 2 ml culture whose apparent absorbance at 730 nm was 0.1 were applied to lanes 1 to 4 which are from the ECT strain incubated in the presence of 1 mM hydrogen peroxide for 0, 24, 48, and 72 h, respectively. In the same SDS-PAGE, samples applied to lanes 5 to 8 are those from the ECT16-1 strain after 0, 24, 48, and 72 h, respectively. In **a**, proteins were stained by CBB and molecular weight markers were separated in lane M. Results shown are from one of the three independent experiments and are representative. L_R^{33}, L_R^{30}, L_C^{27}, PC-β, APC-β, PC-α, and APC-α are the 33 kDa rod linker polypeptide, the 30 kDa rod linker polypeptide, the 27 kDa rod-core linker polypeptide, phycocyanin β subunit, allophycocyanin β subunit, phycocyanin α subunit, and allophycocyanin α subunit. These polypeptides are components of PBSs.

Fig. 4. Protection of the capacity of phycocyanin to absorb light by two small Hsps, α-crystallin and HspA (**a**) and direct interaction of HspA with components of PBS (**b**). 30 μg of PBS in 200 μl of 0.75 M NaK-phosphate (pH 8.0) containing 160 mM hydrogen peroxide were incubated at 30°C for 9 h or 18 h in the absence (No addition) or presence of 30 μg of His-tagged HspA (molecular mass, 19.4 kDa), α-crystallin (20.0 kDa), or lysozyme (14.7 kDa). Each mixture was diluted to 1 ml by the NaK-phosphate buffer for absorption measurements (**a**). The absorbance change was also measured in the mixture containing only PBS (No H_2O_2). For pull down assays (**b**), a mixture containing PBS (331 μg/ml) and/or HspA (132 μg/ml, 6.8 μM) was incubated in 0.75 M NaK-phosphate (pH 8.0) containing 160 mM H_2O_2 at 30°C for 9 h. The His-tagged HspA was precipitated by Ni-chelating resins. Proteins bound to the resin were separated by SDS-PAGE, and stained by CBB R-250. Phycocyanin fluorescence was further detected as described in the legend to Fig. 3. As a reference, PBS that was used for the pull down assays was analyzed in the same way (shown on the right). Abbreviations for components of PBS are given in Fig. 3 legend.

Fig. 5. Cell growth (**a**), viability (**b**), and cellular absorption spectra (**c** and **d**) of the *Synechococcus* ECT and ECT16-1 strains incubated in the presence of 1 μM methyl viologen for 0, 24, 48, and 72 h. Diamonds and squares (**a** and **b**) represent ECT and ECT16-1, respectively. Cells were cultured at 30ºC to approximately 0.2 apparent absorbance at 730 nm. Then, at 0 h methyl viologen was added to cultures to a final concentration of 1 μM. Results shown are representative of three independent growth experiments.

Fig. 6. Cell growth (**a**), viability (**b**), and cellular absorption spectra (**c** and **d**) of the *Synechocystis* sp. strain PCC 6803 (wild type) and its *hspA* deletion mutant strains incubated in the presence of 4 μM methyl viologen for 0, 24, 48, and 72 h. Squares and diamonds (**a and b**) represent the wild type (Wild) and its *hspA* deletion mutant (Δ*hspA*), respectively. Cells were cultured at 30ºC to approximately 0.2 apparent absorbance at 730 nm. Then, at 0 h methyl viologen was added to the culture to a final concentration of 4 μM.

Results in **a** and **b** are averages of three independent experiments; bars indicate standard deviations. **c** and **d** show typical curves obtained in one of three experiments.

Figure legends for supplementary figures

Supplementary Fig. 1. Cell growth (**a**), viability (**b**), and cellular absorption spectra of the *Synechococcus* ECT (**c**) and ECT16-1 (**d**) strains incubated in the presence of 0.8 mM hydrogen peroxide for 0, 24, 48, and 72 h. Diamonds and squares (**a** and **b**) represent ECT and ECT16-1, respectively. Cells were cultured at 30ºC to approximately 0.3 apparent absorbance at 730 nm, and then (at 0 h) hydrogen peroxide was added to a final concentration of 0.8 mM.

Supplementary Fig. 2. Cell growth (**a**), viability (**b**), and cellular absorption spectra of the *Synechococcus* ECT (**c**) and ECT16-1 (**d**) strains incubated in the presence of 0.9 mM hydrogen peroxide for 0, 24, 48, and 72 h. Diamonds and squares (**a** and **b**) represent ECT and ECT16-1, respectively. Cells were cultured at 30ºC to approximately 0.3 apparent absorbance at 730 nm, and then (at 0 h) hydrogen peroxide was added to a final concentration of 0.9 mM.

Fig. 1

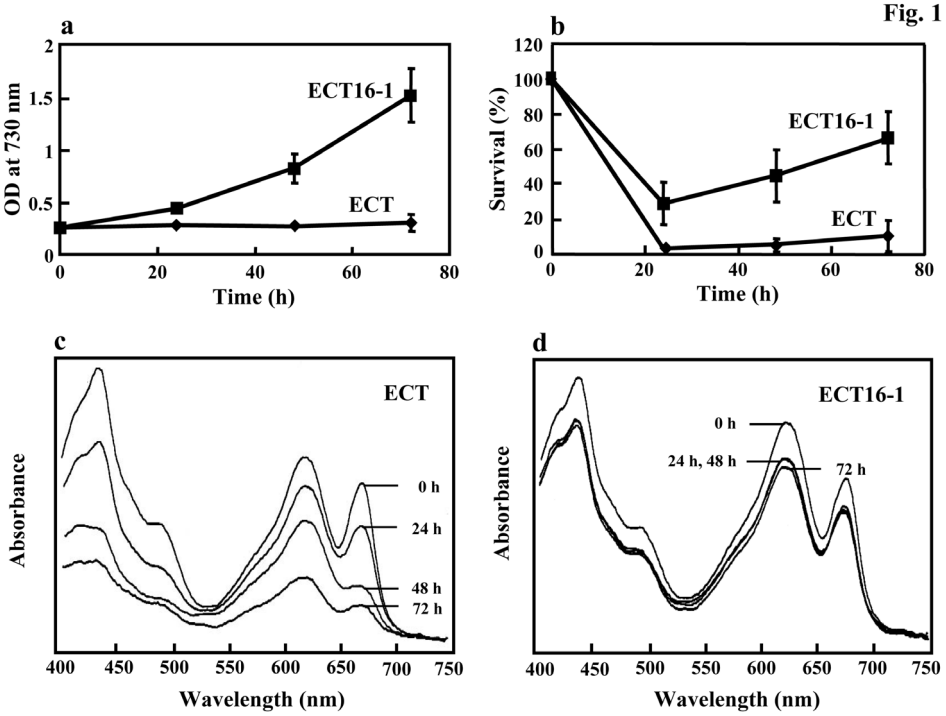

a
OD at 730 nm
Time (h)
ECT16-1
ECT
b
Survival (%)
Time (h)
ECT16-1
ECT
c
Absorbance
Wavelength (nm)
ECT
0 h
24 h
48 h
72 h
d
Absorbance
Wavelength (nm)
ECT16-1
0 h
24 h, 48 h
72 h

Fig.2

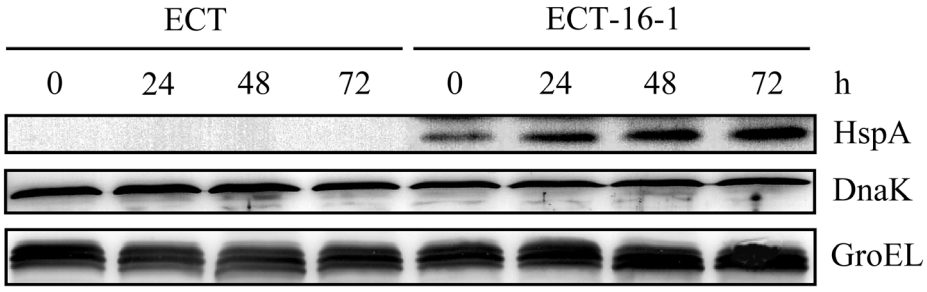


Fig. 3

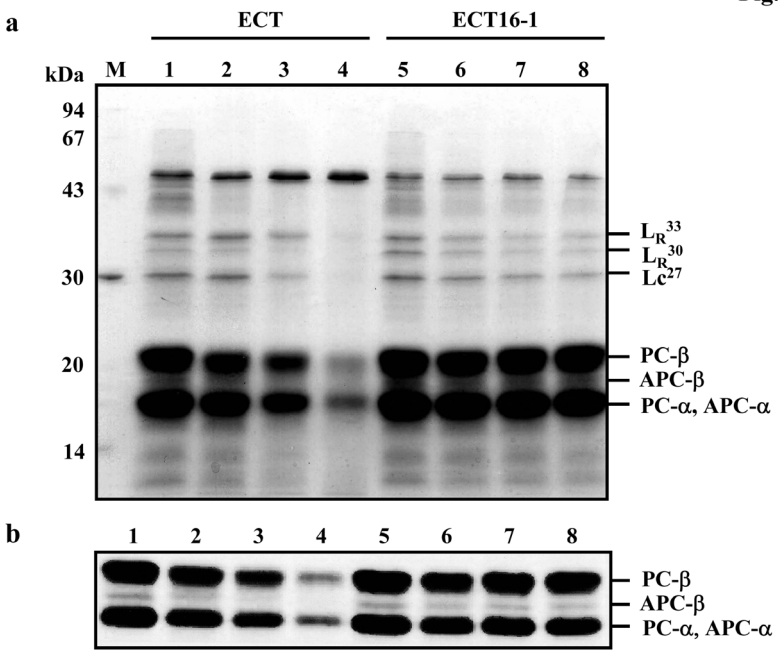

a
ECT
ECT16-1
kDa
M
1
2
3
4
5
6
7
8
94
67
43
30
20
14
L_R^{33}
L_R^{30}
Lc^{27}
PC-β
APC-β
PC-α, APC-α
b
1
2
3
4
5
6
7
8
PC-β
APC-β
PC-α, APC-α

Fig. 4

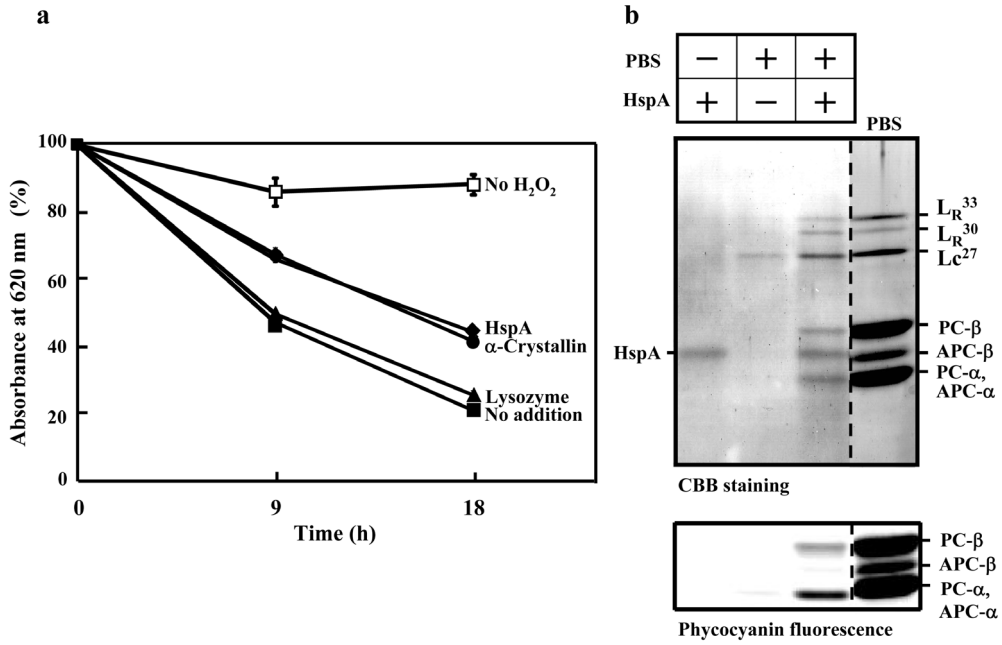

a
b
Absorbance at 620 nm (%)
100
80
60
40
20
0
0
9
18
Time (h)
No H_2O_2
HspA
α-Crystallin
Lysozyme
No addition
PBS
HspA
PBS
HspA
L_R^{33}
L_R^{30}
Lc^{27}
PC-β
APC-β
PC-α,
APC-α
CBB staining
PC-β
APC-β
PC-α,
APC-α
Phycocyanin fluorescence

Fig. 5

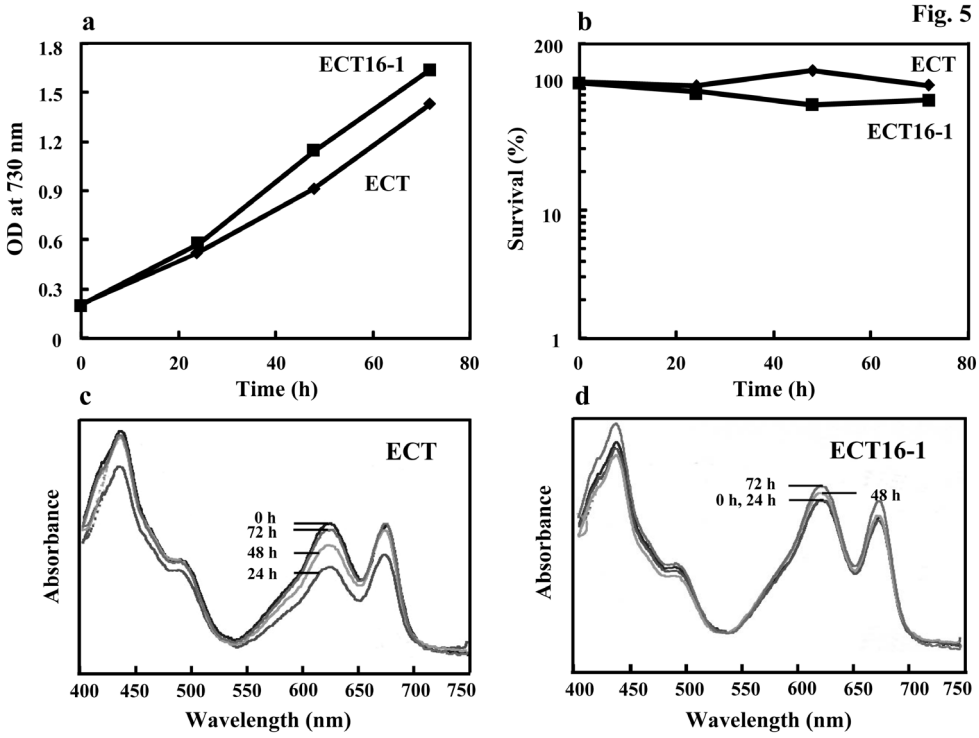
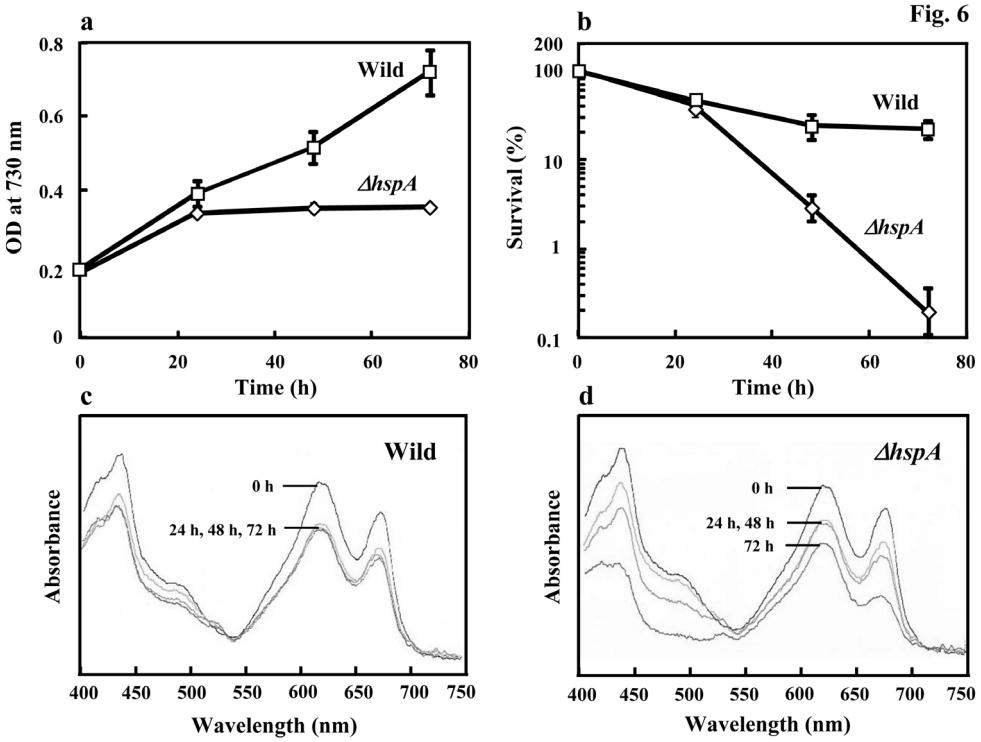
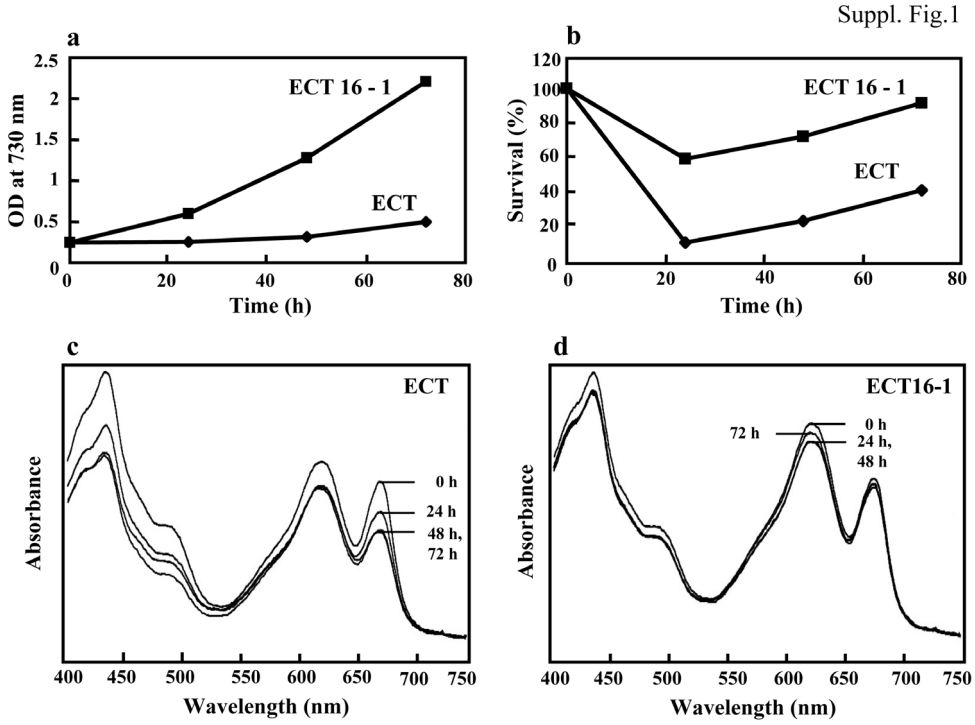

a
ECT16-1
ECT
OD at 730 nm
Time (h)
b
ECT
ECT16-1
Survival (%)
Time (h)
c
ECT
0 h
72 h
48 h
24 h
Absorbance
Wavelength (nm)
d
ECT16-1
72 h
0 h, 24 h
48 h
Absorbance
Wavelength (nm)

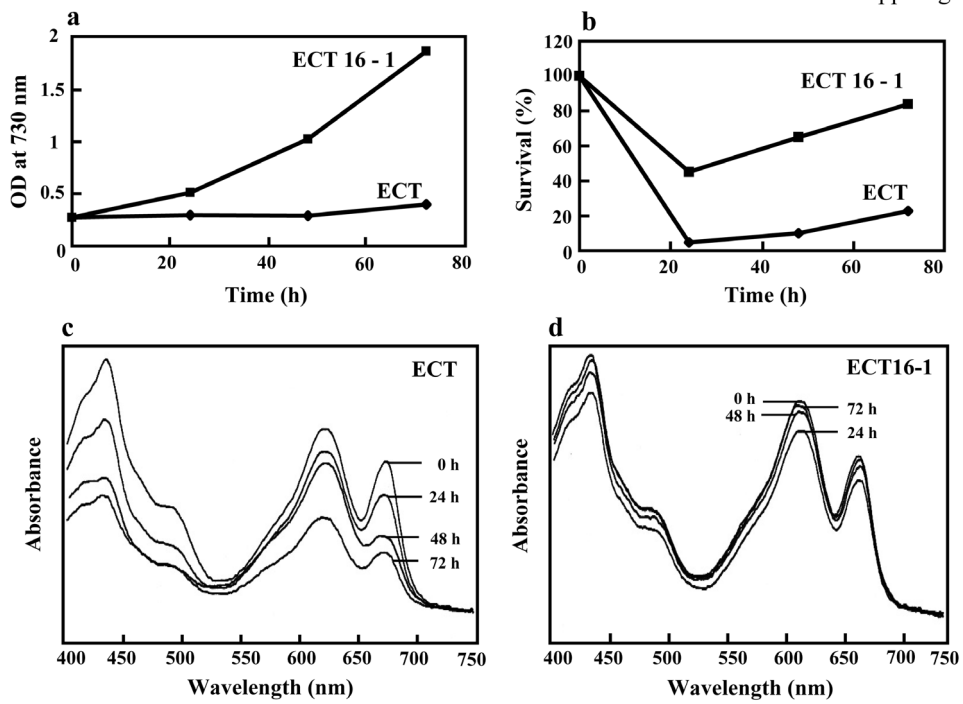
Fig. 6


a
OD at 730 nm
Wild
ΔhspA
Time (h)
b
Survival (%)
Wild
ΔhspA
Time (h)
c
Wild
0 h
24 h, 48 h, 72 h
Absorbance
Wavelength (nm)
d
ΔhspA
0 h
24 h, 48 h
72 h
Absorbance
Wavelength (nm)

Suppl. Fig.1


a
OD at 730 nm
Time (h)
ECT 16 - 1
ECT
b
Survival (%)
Time (h)
ECT 16 - 1
ECT
c
ECT
Absorbance
Wavelength (nm)
0 h
24 h
48 h,
72 h
d
ECT16-1
Absorbance
Wavelength (nm)
72 h
0 h
24 h,
48 h

Suppl.Fig.2


a
OD at 730 nm
ECT 16 - 1
ECT
Time (h)
b
Survival (%)
ECT 16 - 1
ECT
Time (h)
c
Absorbance
ECT
0 h
24 h
48 h
72 h
Wavelength (nm)
d
Absorbance
ECT16-1
0 h
48 h
72 h
24 h
Wavelength (nm)