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Comparative analysis of the *hspA* mutant and wild-type *Synechocystis* sp. strain PCC 6803 under salt stress: evaluation of the role of *hspA* in salt-stress management

Abstract DNA microarray analysis has previously revealed that *hspA*, which encodes a small heat-shock protein, is the second most highly expressed gene under salt stress in *Synechocystis* sp. strain PCC 6803. Consequently, an *hspA* deletion mutant was studied under various salt stresses in order to identify a potential role of HspA in salt stress management. The mutant had a growth disadvantage under moderate salt stress. It lost the ability to develop tolerance to a lethal salt treatment by a moderate salt pre-treatment when the tolerance was evaluated by cell survival and the level of major soluble proteins, phycocyanins, while the wild-type acquired tolerance. Under various salt stresses, the mutant failed to undergo the ultrastructural changes characteristic of wild-type cells. The mutant, which showed higher survival than the wild-type after a direct shift to lethal salt conditions, accumulated higher levels of *groESL1* and *groEL2* transcripts and the corresponding proteins, GroES, GroEL1, and GroEL2, suggesting a role for these heat-shock proteins in conferring basal salt tolerance. Under salt stress, heat-shock genes, such as *hspA*, *groEL2*, and *dnaK2*, were transcriptionally induced and greatly stabilized, indicating a transcriptional and post-transcriptional mechanism of acclimation to salt stress involving these heat-shock genes.

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Introduction

Microorganisms acclimate to various kinds of environmental stress by regulating the expression of stress-inducible genes (VanBogelen et al. 1987; Chuang and Blattner 1993; Hecker et al. 1996; Los and Murata 1999). Cyanobacterial stress genes have been shown to be activated by a variety of stresses, such as heat shock (Borbély et al. 1985; Webb et al. 1990; Roy et al. 1999), nitrogen starvation (Caslake et al. 1997), oxidative stress (Chitnis and Nelson 1991; Hossain and Nakamoto 2003), hyperosmotic stress, and salt stress (Kanesaki et al. 2002).

Salt stress is one of the environmental factors that limit growth and productivity of the organism (Boyer 1982; Garcia-Pichel et al. 1999). Allakhverdiev et al. (2000) investigated the mechanisms of the salt-stress-induced inactivation of the photosynthetic machinery, particularly the oxygen-evolving machinery of the photosystem II complex in *Synechococcus* sp. strain PCC 7942. Salt (0.5 M NaCl) stress has both osmotic and ionic effects that cause, in particular, the influx of Na^+ through K^+ (Na^+) channels with a resultant increase in the intracellular concentration of Na^+ and counter anions, mostly Cl^- (Allakhverdiev et al. 2000). The resultant changes cause irreversible inactivation of the oxygen-evolving machinery. A variety of cyanobacterial defense mechanisms against salt stress have been reported: sucrose is synthesized in salt-sensitive (resistance limit about 0.7 M NaCl) strains of cyanobacteria such as *Synechococcus* sp. strain PCC 6301 (Mackay et al. 1984; Reed et al. 1986; Joset et al. 1996); glucosylglycerol is synthesized in strains with intermediary tolerance (limit about 1.8 M NaCl) such as *Synechocystis* sp.

strain PCC 6803 (Hagemann et al. 1987, 2001; Erdmann et al. 1992; Joset et al. 1996; Mikkat and Hagemann 2000; Marin et al. 2002); and glycinebetaine is synthesized in salt-tolerant (limit about 2.7 M NaCl) *Synechococcus* sp. strain PCC 7418 (Mackay et al. 1984; Reed et al. 1986; Joset et al. 1996).

Kanesaki et al. (2002) have shown, by DNA microarray analysis, that a certain group of genes are strongly induced under salt shock by the presence of 0.5 M NaCl in *Synechocystis* sp. strain PCC 6803. They include genes for proteins involved in translation (*rpl*3) and in the modification and degradation of proteins (*prc* and *ftsH*), heat-shock proteins (*hspA*, *dnaK*, *dnaJ*, *htrA*, *groEL2*, and *clpB*), superoxide dismutase (*sodB*), proteins involved in synthesis of glucosylglycerol (*glpD* and *ggpS*), and sigma 70 factors (2 *rpoD* genes). Induction of genes for heat-shock proteins (HSPs), proteases, and ribosomal proteins under salt shock indicated that the salt shock was associated with a change in the stability of cellular proteins. It has also been demonstrated that DNA, RNA, and protein synthesis in *Synechocystis* cells was affected when cells were incubated in a medium containing 0.684 M NaCl (Hagemann et al. 1994). Cellular protein synthesis drastically dropped immediately after salt shock (0.684 M), was accompanied by qualitative changes, and gradually recovered with time during long-term treatment (Hagemann et al. 1991). The exact mechanism of this recovery remains to be elucidated. One possibility is the synthesis of molecular chaperones and proteases during salt shock in order to avoid the accumulation of denatured proteins and to degrade irreversibly damaged proteins (Kanesaki et al. 2002).

In the present study, the role of *hspA* under salt stress was determined by a comparative analysis of the wild-type *Synechocystis* sp. strain PCC 6803 and its *hspA* null mutant strain. Basal and acquired salt tolerances of the wild-type and the *hspA* mutant strains were examined in order to determine the role of *hspA* in salt stress management. The ultrastructural changes in the two strains under various salt stresses were compared in order to infer the area affected due to the absence of *hspA*. The expression pattern of various HSPs in the *hspA* mutant was compared with that in the wild-type to explain the *hspA* mutant phenotype.

Materials and methods

Organisms and culture conditions

Synechocystis sp. strain PCC 6803 was grown photoautotrophically in BG-11 inorganic liquid medium or on BG-11 plates containing 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate (Rippka et al. 1979). The BG-11 culture medium was modified to contain 50 mg/l Na_2CO_3 and 20 mM *N*-(2-Hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) adjusted to pH 8.0 with KOH. The liquid cultures in glass vessels were incubated at 30°C in a water bath, continuously aerated, and illumi-

nated with a light intensity of 35 μE/m^2/s. The 30 mg/ml rifampicin stock solution was dissolved in methanol. In *Synechocystis* strain HK-1, a kanamycin-resistant *hspA* deletion mutant, the entire *hspA* coding region, as well as 160 bp of downstream sequence, comprising the entire intragenic region between *hspA* and a downstream ORF, was deleted. Correct integration and complete segregation of the mutation in all copies of the genome was confirmed by PCR using the primers shsp-1 (5′-TTCTTTACAATCCCCTGCGG-3′) and shsp-2 (5′-TAATACGACTCACTATAGGGTCAAAGTTAGGA-TACCGGCAT-3′).

Salt-shock conditions

An appropriate volume of a 3.0 M NaCl was added to a culture of cells growing exponentially to the final concentration of 0.5 M and the culture was incubated under the standard growth conditions (at 30°C, 35 μE/m^2/s) for 10 min unless otherwise stated. In the case of heat shock, a culture was transferred to a pre-warmed glass vessel in a 42°C water bath, and continuously aerated under the same light intensity.

Viability assays

Cell survival was determined as described previously (Nakamoto et al. 2000).

Transmission electron microscopy

The ultrastructural changes of the wild-type and *hspA* mutant cells of *Synechocystis* sp. strain PCC 6803 were studied under salt stress at a salt concentration of 2.0 M. The treatment time (12 h) was chosen after determining the cell survival rate at different time intervals up to 24 h. The desired salt concentration was achieved by adding crystalline NaCl to the culture. For glutaraldehyde fixation, 8% glutaraldehyde was added to the culture tube directly drop by drop until a final concentration of 2% was reached. Glutaraldehyde fixation was carried out at room temperature for 1 h and in the refrigerator (4°C) overnight. After glutaraldehyde fixation, specimens were precipitated by centrifugation and rinsed with 0.05 M potassium phosphate buffer (pH 7.0) for 1 h. The pellets were then post-fixed with 2% osmium tetroxide in the buffer for 1 h at room temperature. The samples were rinsed with buffer for 10 min, then dehydrated gradually in an acetone series (10, 30, 50, 70, 85, 95, and 100%; 10 min for each step). Dehydrated samples were immersed in equal parts of propylene oxide and acetone for 20 min and then in 100% propylene oxide for 20 min. The samples were then stepwise infiltrated with Spurr's resin. The resin was polymerized in an oven, at 70°C overnight. Ultrathin sections (silver-gold) were cut using a

diamond knife on a Sorvall MT-2B ultramicrotome. After staining with 2% uranyl acetate for 10 min and with lead citrate for 5 min, sections were observed with a Hitachi H-7500 electron microscope at 100 kV. For each treatment, micrographs of more than 100 cells were examined.

Fluorescence microscopy

Fluorescence microscopic images were obtained using a Nikon LABOPHOTO microscope (Japan) equipped with an Hg lamp (HBO 50). Images were acquired at 1,000× magnification using a digital KEYENCE VB 6000/6010 camera (Japan). Samples were examined using a 420 nm filter in the case of DAPI (DNA Probe) staining, and a 515 nm filter in the case of SYBR Gold (DNA Probe) staining.

Preparation of total RNA

Cells that had been exposed to various experimental conditions were immediately collected by centrifugation and frozen in liquid N_2. Alternatively, cells were killed instantaneously by the addition of an equal volume of 10% phenol in ethanol to the cell suspension, collected by centrifugation, and frozen in liquid N_2 (Kanesaki et al. 2002). Total RNA was extracted as described previously (Nakamoto and Hasegawa 1999).

Northern blot analysis

The RNA preparation was loaded for each lane of a 1% agarose gel as stated in each figure legend. After electrophoresis, RNA was blotted onto the BM positively charged nylon membrane (Boehringer Mannheim), and cross-linked. A separate blot was simultaneously hybridized with a different probe following the manufacturer's instructions (Boehringer Mannheim). Digoxygenin-labeled RNA probes were hybridized as described previously (Asadulghani et al. 2003; Nakamoto et al. 2003).

Protein extraction

Cultured cells were harvested by centrifugation at room temperature for 5 min at 3,000 rpm in a Beckman GS-6 table-top centrifuge. The cell pellet was collected and used immediately. The pellet was washed with TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and resuspended in TE containing 1 mM each of phenylmethylsulfonyl fluoride, benzamidine and caproic acid. Subsequently, cells were ruptured by a French press (at 20,000 PSI) and incubated with 1% Triton X-100 and 3 M urea on ice for 1 h. Finally the resulting suspension was centrifuged at 4°C and 16,000g for 30 min. The

supernatant fraction was used for SDS-PAGE, Western blot analysis and two dimensional polyacrylamide gel electrophoresis (2D-PAGE).

Gel electrophoresis and Western blot analysis

Equal amounts of crude soluble protein were boiled for 5 min in a mixture containing 50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol and then loaded onto a 15% polyacrylamide gel in the presence of SDS (Roy et al. 1999). Western blot analysis was performed to detect GroES, GroEL and DnaK in cell extracts by using anti-*Synechococcus vulcanus* polyclonal antibodies as probes (Roy et al. 1999). 2D-PAGE was carried out by the method of O'Farrell (1975), using Immobilin dry strip, pH 3–10 (Amersham Pharmacia).

Results

Effect of salt stress on the growth and survival of the wild-type and the *hspA* mutant

DNA microarray analysis has revealed salt stress at 34°C to be a strong enhancer of the expression of several HSP genes, such as *clpB*, *dnaK2*, *groEL2* and *hspA*, in the cyanobacterium *Synechocystis* sp. strain PCC 6803 (Kanesaki et al. 2002). The expression of *hspA*, which encodes a small HSP (sHSP) homolog, was the second most strongly enhanced among 147 ORFs that appeared above the upper reference line, while the highest enhancement occurred in a gene of unknown function (Kanesaki et al. 2002). In the present study, the phenotype of the *hspA* null mutant strain HK-1 was analyzed with the aim of assigning a salt-stress-related function for this gene.

First, growth of the *hspA* mutant was compared with that of the wild-type at 30°C under normal light intensity, 35 $\mu E/m^2/s$, in the presence of either 0.5 M or 2.0 M NaCl by measuring the OD at 730 nm. No significant difference was observed in the growth rate at 0.5 M NaCl (Fig. 1a), while 2.0 M NaCl completely inhibited the growth of both strains (data not shown). Under a higher light intensity, 100 $\mu E/m^2/s$, in the presence of 0.5 M NaCl, both strains showed a 40–45% reduction in the growth rate until 24 h of incubation. However, the wild-type growth rate recovered to the level in the absence of NaCl after 72 h incubation, whereas the *hspA* mutant growth rate did not (Fig. 1a). The results suggest that the *hspA* mutant cannot acclimate to the salt-stress conditions, although the wild-type can within 24 h.

It is well known that cells can acquire tolerance to lethal stress by a moderate pretreatment of the same type of stress. In order to quantify the ability of both strains to acclimate to salt stress, the number of surviving cells was compared after a lethal salt (2.0 M
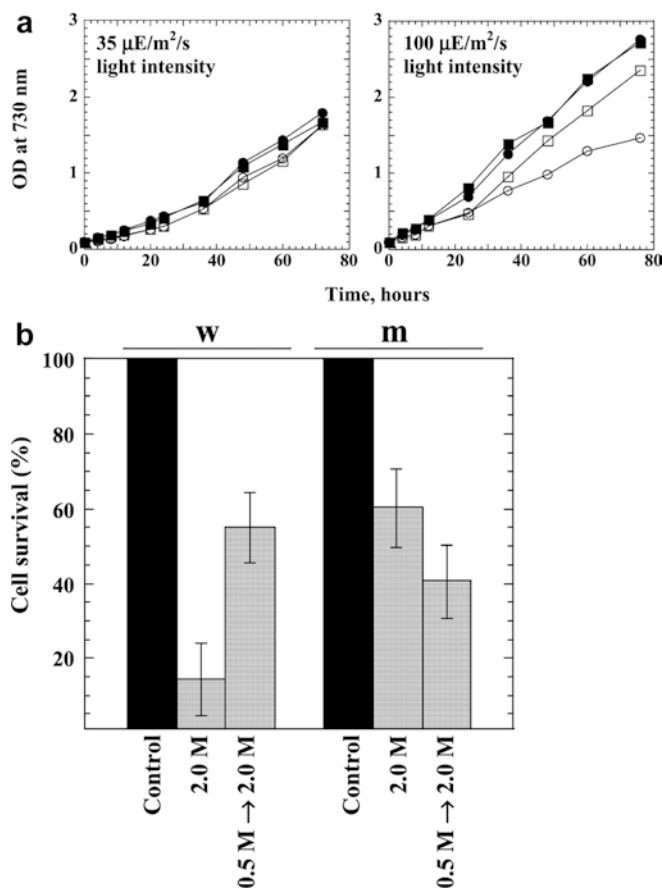
Fig. 1 Growth and survival of wild-type and *hspA* mutant cells under salt-stress conditions. **a** Growth of wild-type (*squares*) and mutant cells (*circles*) at 30°C and at a light intensity of either 35 μE/m^2/s or 100 μE/m^2/s, in the absence (*filled symbols*) or presence (*open symbols*) of 0.5 M NaCl. **b** Survival of the wild-type (*w*) and mutant (*m*) cells after a shift to 2.0 M NaCl for 12 h with (0.5 M → 2.0 M) or without (2.0 M) a 0.5 M NaCl pre-treatment for 2 h. The number of cells before the shift to 0.5 or 2.0 M NaCl treatment (control) was taken as 100%. Values from three independent experiments are shown (means ± SD)

NaCl) treatment with or without a moderate salt (0.5 M) pretreatment (Fig. 1b). The concentration of NaCl was increased by adding solid NaCl into the culture. After a direct shift to 2.0 M NaCl for 12 h, 13% of the wild-type cells survived, whereas 60% of the *hspA* mutant cells were still viable. Further experiments to reveal a possible mechanism for the high basal salt tolerance of the mutant are described below. When a 12-h lethal salt treatment of wild-type cells was preceded by a 2-h moderate salt pre-treatment (0.5 M → 2.0 M), cell survival was increased to 55% of the initial cells before the salt stress. Thus, the wild-type cells acquired salt tolerance through the pretreatment. As far as we know, this is the first demonstration of acquired salt tolerance of a cyanobacterium by a moderate salt pretreatment. By contrast, the survival rate of the *hspA* mutant cells after the same pretreatment decreased to 40%, indicating that the *hspA* mutant did not acquire salt tolerance. Individual experiments, from which the average was

calculated as shown in Fig. 1b, revealed that the survival rate of the mutant strain after the pretreatment never exceeded the level after the direct shift to the lethal salt stress. Similar results were also observed with different incubation times (2-h or 6-h instead of 12-h) for lethal salt stress (data not shown). These results show that the *hspA* mutant failed to acquire salt tolerance from moderate salt stress.

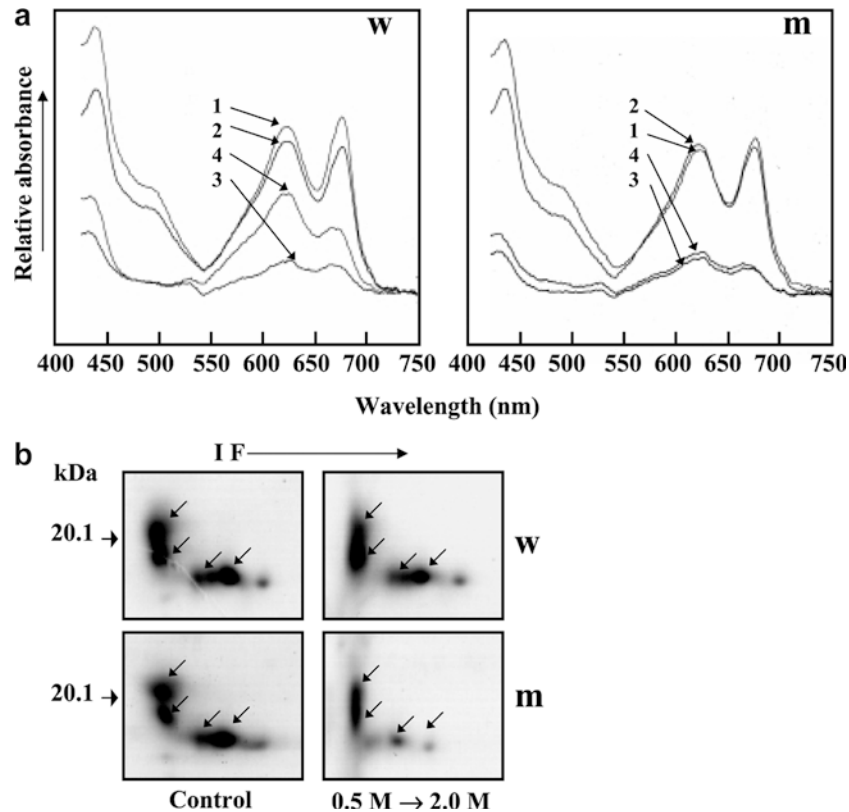### Effect of salt stress on the stability of phycobiliproteins in the wild-type and the *hspA* mutant cells

Since a change in the coloration of the wild-type and the *hspA* mutant cells incubated in the presence of 2.0 M NaCl was observed, the absorption spectra of the cells were analyzed (Fig. 2a). Although there was no significant effect of the incubation of cells in the presence of 0.5 M NaCl on the spectra of the wild-type and *hspA* mutant cells, a direct shift of the cells to 2.0 M NaCl led to major decreases in absorption peaks of phycocyanin (620 nm) and chlorophyll a (430 and 670 nm). However, bleaching in the wild-type cells with a moderate salt pretreatment was significantly delayed (Fig. 2a), whereas the same pre-treatment did not retard the reduction of these photosynthetic pigments in the *hspA* mutant.

In order to confirm that the decrease in the 620-nm absorption peak was caused by the reduction of the phycobiliprotein level and to examine which phycobiliproteins decreased, 2D-PAGE analysis was carried out. The analysis of the protein samples from the salt-treated cells revealed that the level of different phycobiliproteins in the wild-type strain after a moderate salt pretreatment followed by lethal salt stress (0.5 M → 2.0 M) was similar to the level in the absence of NaCl, whereas the level in the *hspA* mutant cells was around one third of the level in the absence of NaCl (Fig. 2b). The levels of all phycobiliproteins were reduced significantly. These results clearly indicate that the decrease in the 620-nm absorption peak shown in Fig. 2a is due to the decrease in the phycobiliprotein level.

Results shown in Figs. 1, 2 indicate that the *hspA* mutant has lost its ability to develop salt-stress tolerance.

Ultrastructural changes in the wild-type and the *hspA* mutant strain under various salt stresses

Subsequently, the ultrastructure of cells that had been used for the quantitation of cell survival was examined by transmission electron microscope (TEM) in order to find out whether there were differences related to the variation of cell survival after the salt treatments. Both wild-type and *hspA* mutant cells showed a typical ultrastructural composition (Stainer 1988; Jensen 1993; Zuther et al. 1998; Lee et al. 2000) when the cells were grown at 30°C, but, in general, the *hspA* mutant cells were smaller than the wild-type cells (Fig. 3a,b). Electron-dense osmiophilic granules, lipid inclusions (Jensen

Fig. 2 Effect of salt stress on the stability of phycocyanin in the wild-type and the *hspA* mutant cells. **a** Absorption spectra of the wild-type (*w*) and mutant (*m*) cells incubated at 30°C in the light (35 µE/m²/s) for 72 h in the presence of 2.0 M NaCl preceded by incubation in the presence (*line 4*) or absence (*line 3*) of 0.5 M NaCl for 72 h. The spectra of cells growing under standard conditions at 30°C (*line 1*) and cells incubated in 0.5 M NaCl for 72 h (*line 2*) are also shown. Whole-cell absorbance was measured at room temperature with a Hitachi 557 dual wavelength double beam spectrophotometer (Hitachi Koki,Tokyo,Japan). **b** Accumulation of different phycobiliproteins in the wild-type (*w*) and mutant (*m*) strains in samples grown at 30°C (control), and after a shift to 2.0 M for 12 h preceded by incubation in 0.5 M NaCl for 2 h (0.5 M → 2.0 M). Different phycobiliproteins are marked by *arrows*. Two-dimensional gel electrophoresis was carried out using 60 µg proteins from cell extracts. The *arrow* at the top of the two dimensional gel electrophoresis photos indicates the direction of isoelectric focusing (I F)

1993), were more evenly distributed in wild-type cells and the number of these granules was higher (Fig. 3a, w1, w2, and w3). After salt treatments, the number of granules in the *hspA* mutant cells was significantly reduced compared to the wild-type cells (Fig. 3b, m1, m2, and m3). In salt-treated cells, thylakoid membrane structure tended to be more clearly discernible in the *hspA* mutant than in the wild-type (Fig. 3a,b). In the stressed cells, the centers were completely devoid of cytoplasm and contained aggregated fibrous structures. These structures could be stained by either DAPI or SYBR Gold (Molecular Probes) and detected under the fluorescence microscope at DNA-specific wavelengths, confirming that they were aggregated DNA fibers (Fig. 3c). In unstressed cells, aggregated fibrous struc-

tures were not observed (Fig. 3a, w1; b, m1). Cytoplasmic structure was least preserved after the lethal salt treatment in the wild-type cells (Fig. 3a, w2). The most conspicuous differences between the two strains during the treatments were in the degree of aggregation of centric fibrous structures and the appearance of dilated thylakoid membrane structures. In the case of the wild-type cells after a direct lethal salt treatment (Fig. 3a, w2), thylakoid membranes were severely affected and instead of regularly spaced parallel membranes dilated areas (marked with double arrows) appeared along the membranes more frequently than in the *hspA* mutant (Fig. 3b, m2). Dilated thylakoid membranes were observed in some *hspA* mutant cells after direct lethal salt stress but not as frequently as in wild-type cells. Moreover, after lethal salt treatment, wild-type cells showed a higher degree of aggregation in the centric fibrous structures compared with the *hspA* mutant (Fig. 3a, w2; b, m2). The evenly spaced, parallel thylakoid membrane structure observed during stepwise increase to lethal salt concentration indicated acclimation both in wild-type and mutant cells compared to the direct lethal treatment (Fig. 3a, w3; b, m3), but the *hspA* mutant failed to reduce the tendency of aggregating the centric fibrous structures as in the case of the wild-type cells. These results indicate that the orderly state of cellular lipids in the *hspA* mutant cells even under lethal salt stress conferred a relatively high tolerance to the stress, and the increase of disorder in the centric DNA fibrous body affected the cellular status under salt stress causing a reduction in cell survival.
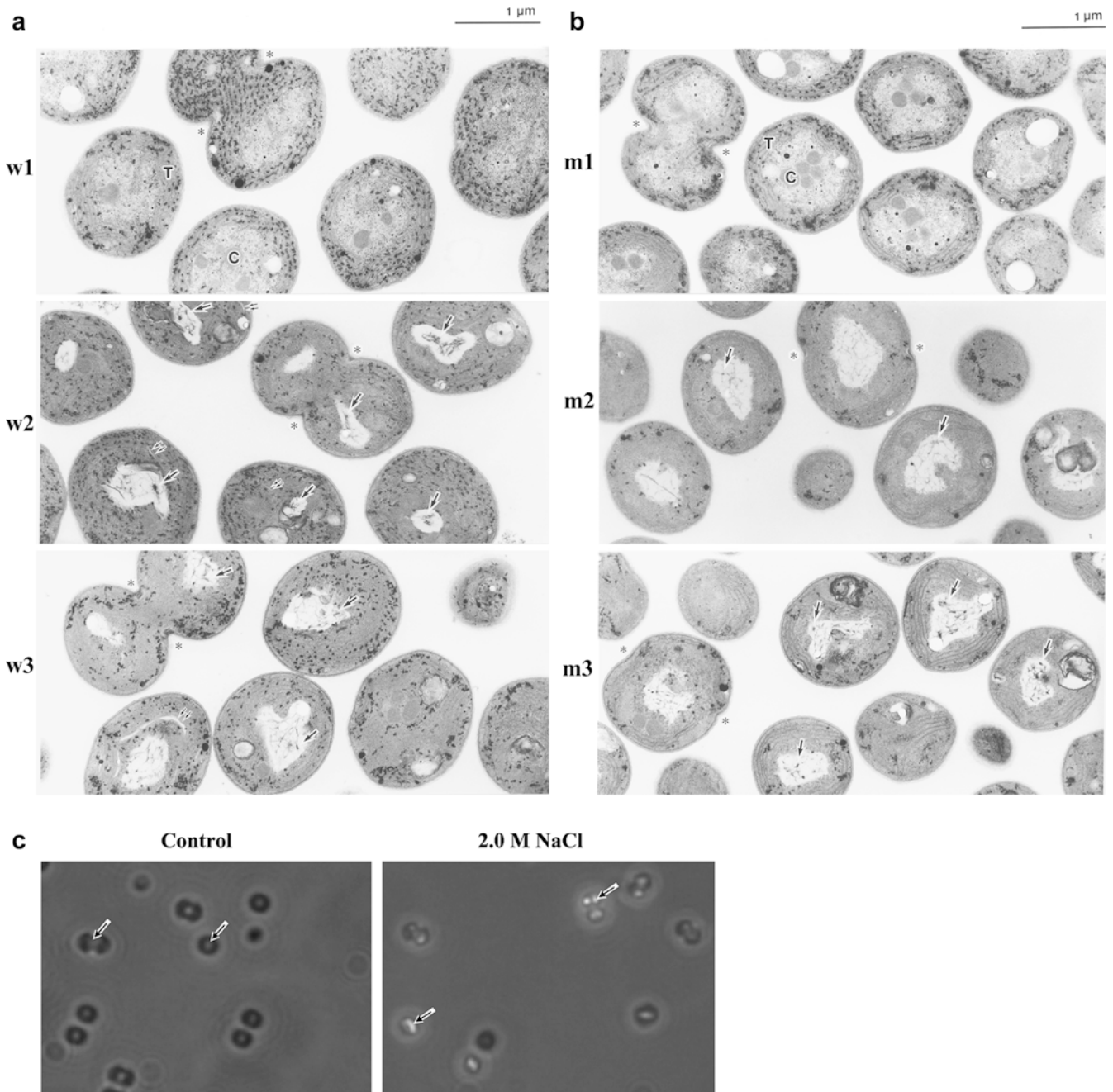
Fig. 3 Ultrastructural changes in the wild-type and the *hspA* mutant strain under various salt stresses. **a** Transmission electron micrographs of wild-type (*w*) cells under various salt stresses. Cells grown at 30°C in the light (35 μE/m²/s) (*w1* and *m1*), either directly treated with 2.0 M for 12 h (*w2* and *m2*) or initially treated with 0.5 M for 2 h and then to 2.0 M for 12 h (*w3* and *m3*) in the light at 30°C. Thylakoid membrane (*T*), carboxysome (*C*), dilated thylakoid membrane (*double arrow*), aggregated fibrous structures (*thick arrow*) are shown in the figure. Cells undergoing division are indicated by an (*asterisk*) on either side of the septum formation. **b** Electron micrographs of the *hspA* mutant (*m*) cells after similar treatment as for the wild type. **c** Wild-type cells grown at 30°C in the light (35 μE/m²/s) were treated with 2.0 M NaCl for 12 h at 30°C and analyzed under fluorescence microscope after staining with SYBR Gold. Stain uptake of cells before (control) or after the lethal salt treatment (2.0 M NaCl) is indicated by *arrows*

Effect of salt stress on the transcriptional and post-transcriptional regulation of heat shock genes

Analysis of the cell survival and ultrastructural changes showed some intriguing phenomena in the *hspA* mutant. The *hspA* mutant showed higher basal salt tolerance than the wild-type (Fig. 1b). Individual experiments, from which the average was calculated as shown in Fig. 1b, showed that the survival rate of the mutant strain after a direct shift to 2.0 M NaCl always exceeds that of the wild-type. It is not likely that the higher salt tolerance of the mutant is due to the presence or absence of HspA since the wild-type does not express *hspA* sig-

nificantly under normal growth conditions (Fig. 4). We speculate that the deletion of *hspA* induced a compensatory response for the absence of HspA. The response includes enhancement of expression of other HSPs. DnaK plays a role under salt-stress conditions in the halo-tolerant cyanobacterium *Aphanothece halophytica* (Hibino et al. 1999). We therefore examined whether the absence of *hspA* had an effect on the expression of other HSP genes under normal and salt-stress conditions.

Figure 4a shows the accumulation of different HSP mRNAs after incubation of the *hspA* mutant and wild-type cells under normal conditions at 30°C in the pres-
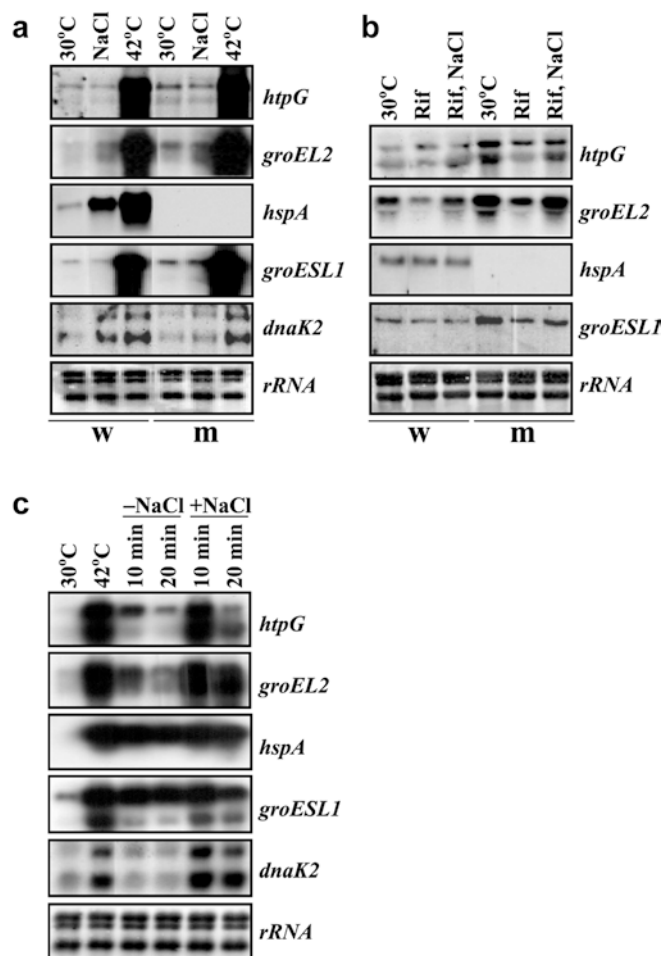
Fig. 4 Effect of salt stress on the transcriptional and post-transcriptional regulation of heat shock genes. **a** Accumulation of different HSP mRNAs in the wild-type (*w*) and the *hspA* mutant (*m*) strains, before (30°C) or after heat shock at 42°C for 10 min (42°C). The levels of mRNAs after incubation at 30°C for 10 min in the presence of 0.5 M NaCl are also shown. **b** Stabilization of the *htpG* and *groEL2* mRNAs of the wild-type (*w*) and the *hspA* mutant (*m*) cells in the presence of 0.5 M NaCl. Rifampicin (300 μg/ml) was added 2 min prior to incubation for 10 min at 30°C, either in the absence (Rif) or presence (Rif, NaCl) of 0.5 M NaCl. **c** Effect of NaCl (0.5 M) on the posttranscriptional stability of HSP mRNAs at 30°C in the wild-type cells after heat induction. Cells growing at 30°C were heat-shocked at 42°C for 10 min and subsequently incubated with 300 μg rifampicin/ml in the absence (−NaCl) or presence (+NaCl) of 0.5 M NaCl for 10 min and 20 min. The amount of RNA loaded (**a** 1 μg, **b** 4 μg; and **c** 1 μg) was verified by the rRNA staining level

ence of 0.5 M NaCl. Reproducibly, the *hspA* mutant showed elevated levels of the two *groE* and *htpG* mRNAs as compared with the wild-type. Salt stress induced the accumulation of *groEL2*, *hspA* and *dnaK2* transcripts in the wild-type at the normal growth temperature, while considerably higher basal levels of the *groESL1* and *groEL2* transcripts in the *hspA* mutant strain were not further increased by salt shock. Although the induction of *groEL2* under salt stress in the wild-type is in accordance with the DNA microarray analysis (Kanesaki et al. 2002), the level of the mRNA accumulation is much less than that under heat-shock conditions (Fig. 4a). At least two RNA bands were found in each repeated experiment for *htpG*, *groESL1*, *groEL2*, and *dnaK2* transcripts, which may have been generated by segmental degradation of the transcripts or by the transcription initiation and/or termination at multiple sites (Asadulghani et al. 2003; Nakamoto et al. 2003).
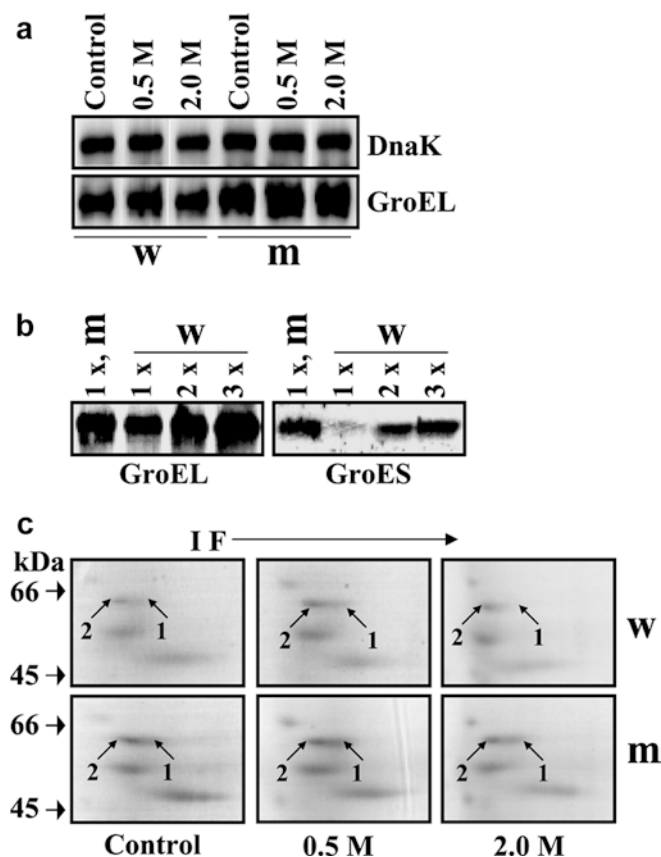
Marin et al. (2002) observed that salt stress stabilizes not only salt-induced transcripts but also non-salt-induced transcripts while activating the de novo synthesis of the transcripts of the saltstress-responsive gene *ggpS* and blocking the transcription of the non-responsive gene *psbC*. We examined whether salt stress has a similar effect on salt-shock-induced heat-shock genes. Rifampicin was added to cultures of the wild-type and the *hspA* mutant strains at 30°C and incubated for 2 min. Immediately after the rifampicin treatment, the culture was further incubated for 10 min with or without the addition of 3.0 M NaCl to a final concentration of 0.5 M. Our results show that the *htpG* and *groEL2* mRNAs are stabilized by salt stress (Fig. 4b). A similar result was also observed in the case of *dnaK2* transcripts (data not shown). In the case of the *hspA* mutant, as there was a considerably higher level of constitutively accumulated transcripts of *htpG* and *groEL2*, the observed effect was clearer.

The above results were further confirmed with heat-shocked wild-type cells, which accumulate much higher levels of mRNAs of various heat-shock genes than do cells under normal conditions. Cells heat-shocked at 42°C for 10 min were incubated at 30°C in the presence of 300 μg rifampicin/ml with or without 0.5 M NaCl. Even after 20 min incubation in the presence of 0.5 M NaCl, considerable levels of *groEL2* and *dnaK2* transcripts were found. However, mRNAs of these heat-shock genes were reduced to the basal levels in cells incubated without NaCl (Fig. 4c). Interestingly enough, non-salt-induced *htpG* mRNA was also stabilized. No significant effect was observed in the case of *groESL1* and *hspA* transcripts since these mRNAs were much more stable than the *htpG*, *groEL2*, and *dnaK2* mRNAs in the absence of NaCl.

Elevated levels of GroES, GroEL1, and GroEL2 in the *hspA* mutant

Transcriptional analysis showed that expression of *groESL1* and *groEL2* is enhanced under normal condi-

tions in the *hspA* mutant, and that *groEL2* mRNA is greatly stabilized in the presence of NaCl. In accordance with the results of transcriptional analysis, the *hspA* mutant cells accumulated higher levels of GroEL compared with the wild type under both stress and nonstress conditions, and the accumulation of DnaK was rather similar in the two strains, after various salt treatments (Fig. 5a). These results suggest that a compensatory mechanism for the acclimation to salt stress is provided by the higher levels of accumulation of GroEL and that the absence of *hspA* affected the regulation of *groE* expression by an unknown molecular mechanism. The extent of the increased levels of GroES and GroEL

in the *hspA* mutant as compared with those in the wild-type cells under normal growth conditions was quantitated (Fig. 5b). Reproducibly, accumulation of GroES and GroEL levels in the *hspA* mutant was at least twice as high . The increase in the GroES level indicates that GroEL1 level also increases in the mutant.

The polyclonal antibodies crossreacted with both GroEL1 and GroEL2, and the sizes of the two proteins were similar; a differential effect of salt stress on the accumulation of the two GroEL proteins was thus not observed by Western blot analysis after SDS-PAGE. The differential effect of various salt stresses on the accumulation of the two GroEL proteins was evaluated by 2D-PAGE (Fig. 5c). Kovács et al. (2001) separated the two chaperonins GroEL1 and GroEL2 on 2D-PAGE. We found an increase in the level of GroEL2 in both the wild-type and the *hspA* mutant after a 0.5 M NaCl incubation for 2 h (Fig. 5c), suggesting that salt stress preferentially facilitates the accumulation of GroEL2 rather than GroEL1. In support of this hypothesis, there was no increase in the GroES level after the 0.5-M NaCl incubation in either strain (data not shown). The increased basal levels of GroEL1, GroEL2, and GroES in the *hspA* mutant during the lethal salt stress (Fig. 5a–c) must therefore be vital for providing a high degree of basal salt tolerance as reflected by the survival of the organism after a direct shift to the lethal salt stress (Fig. 1b).

Discussion

In the present study, two *Synechocystis* strains, the *hspA* null mutant strain HK-1 and the wild-type PCC 6803, were compared under various salt-stress conditions with respect to cell growth, survival, phycobiliprotein level, and ultrastructural changes, and significant differences were found. The transcriptional and posttranscriptional effect of salt shock on heat-shock gene expression in the *hspA* mutant and in the wild-type strain was subsequently analyzed in order to find the reasons for the observed differences. The results presented here give evidence that *hspA* is involved in salt-stress management. The results also suggest that GroEL and GroES play a compensatory role in the *hspA* mutant, which is not unlikely as GroEL and HspA share similar functions although in different modes (Török et al. 1997; Horváth et al. 1998; Török et al. 2001; Kenneth et al. 2004). In addition, salt shock was found to have a dual effect on the expression of HSP genes, that is, activation of transcription of *hspA*, *groEL2*, and *dnaK2*, and an increase in the posttranscriptional stability of *groEL2*, *dnaK2*, and *htpG* mRNAs (Fig. 4a–c).

Bacteria possess mechanisms to adapt to sublethal stresses, rendering them resistant to lethal levels of the same stress. Heat-shock genes such as *groEL2*, *hspA*, and *dnaK2*, are induced during sublethal salt (0.5 M NaCl) treatment of the wild-type strain (Fig. 4a), and they may play a role in providing salt tolerance to cells

Fig. 5 Elevated levels of different HSPs in the *hspA* mutant (*m*) and in the wild-type (*w*). **a** Western blot analysis for 10-μg protein samples in each lane. GroEL and DnaK were specifically detected using polyclonal antibodies raised against each protein from the cyanobacterium *Synechococcus vulcanus*. The polyclonal antibodies raised against GroEL cross-react with both GroEL1 and GroEL2. **b** Western blot analysis of proteins (5 μg) from the mutant (1×, *m*) and 5 μg (1×), 10 μg (2×), and 15 μg (3×) protein from the wild-type (*w*) cells grown under the standard growth conditions at 30°C. GroEL and GroES were specifically detected using polyclonal antibodies raised against each protein from *Synechococcus vulcanus*. **c** Two-dimensional gel electrophoresis of proteins (60 μg) showing the accumulation of GroEL2 (*2*) and GroEL1 (*1*) stained with Coomassie Brilliant Blue (G250). Proteins were extracted from cells grown at 30°C in the light (35 μE/m²/s) (control), after incubations in 0.5 M NaCl for 2 h (0.5 M) and 2.0 M NaCl for 12 h (2.0 M). The *arrow* on the top of the two-dimensional gel electrophoresis photo indicates the direction of isoelectric focusing (I F)

(Figs. 1, 2). Wild-type cells may benefit from the presence of *hspA* since the *hspA* mutant lost the capacity to acquire salt tolerance (Fig. 1b). However, the high basal level of *groEL2*, *groESL1*, and *htpG* expression (Figs. 4, 5) may bestow a higher basal salt tolerance on the mutant than on the wild-type, as was revealed when both strains were subjected to a direct lethal salt challenge (Fig. 1b).

Previous reports concerning the functional aspects of *hspA* imply a role for this gene in the development of thermotolerance (Lee et al. 1998, 2000) and in the maintenance of membrane physical order (Horváth et al. 1998). In vitro, sHSPs were found to bind nonnative proteins in order to prevent their irreversible aggregation by maintaining them in a refolding competent state, which thus provides a reservoir of stably unfolded proteins and allows interaction with ATPase chaperones to restore these inactive proteins to a functional state (Ehrnsperger et al. 1997; Lee et al. 1997; Veinger et al. 1998; Haslbeck et al. 1999; Lee and Vierling 2000). Like other sHSPs, recombinant *Synechocystis* HspA was found to serve as a reservoir for the unfolded substrate, transferring it to the DnaK/DnaJ/GrpE and GroES/GroEL chaperone network for subsequent refolding (Török et al. 2001). It is plausible that salt-induced damage to the cellular proteinaceous machinery involves both unfolding and irreversible aggregation, in which HspA prevents unfolded proteins from irreversible aggregation by maintaing them in a folding-competent state and supplying them to ATPase chaperones, such as GroES/GroEL. Thus, HspA and GroES/GroEL may play a key role in the suppression of irreversible protein aggregation in cells after a long-term salt treatment. The collaboration between HspA and GroES/GroEL in protecting proteins from irreversible denaturation may be essential for the acquisition of salt tolerance, since the *hspA* mutant lost the acquired salt tolerance (Fig. 1b). One of the target proteins for HspA may be phycobiliproteins, and HspA may interact with phycobiliproteins directly or indirectly to protect them under salt stress. Thus, phycobiliproteins in the *hspA* mutant are highly unstable even after a pre-salt-treatment which induces HspA and GroEL2 in the wild-type in order to stabilize phycobiliproteins.

Comparative analysis of the electron micrographs showed that the *hspA* mutant cells failed to undergo the changes in ultrastructure characteristic of wild-type cells under salt stress, suggesting a role for *hspA* in salt-stress management (Fig. 3). Tsvetkova et al. (2002) demonstrated that HspA can regulate membrane lipid polymorphism during thermal stress. Under salt stress, a change in the ultrastructure of cyanobacterial thylakoid membranes may also require the modulation of membrane lipid polymorphism for recovery. In fact, salinity affects several physical and chemical features of membranes with resulting increase in fluidity (Joset et al. 1996), and *hspA* was identified as a fluidity gene whose expression is enhanced by the administration of a fluidizer (Horváth et al. 1998). Similar to HspA, GroEL-GroES chaperonin heterooligomers are able to interact with membrane lipids. They assist in the folding of both soluble and membrane-associated proteins and concomitantly stabilize the lipid membrane structure (Török et al. 1997). Thus, the enhanced expression of *groESL1* and *groEL2* in the *hspA* mutant may compensate for the lack of HspA that plays a role in membrane quality control.

In the present study, we showed for the first time that salt stress induces aggregation of cellular DNA, suggesting a role of HspA in controlling accumulation and aggregation of centric DNA fibers under salt-stress conditions. HspA may interact with proteins involved in the formation of the nucleoid. An inhibition of cell division and an increase in cell size were reported in the wild-type *Synechocystis* sp. strain PCC 6803 at high concentrations of NaCl (Ferjani et al. 2003). The correlation between inhibition of cell division and aggregation of DNA fibers, and the involvement of HSPs in these processes remains to be elucidated.

Although salt shock inhibits photosynthetic activity to a greater extent in *Synechocystis* sp. strain PCC 6803 compared to other stresses, such as heat shock or intense light (Fulda et al. 1999; Allakhverdiev et al. 1999), the activation of *hspA* and *groEL2* transcription took place under salt stress, indicating that the transcription of these genes can occur independently of photosynthetic activity (Fig. 4a). Our previous study indicated that transcriptional activation of *hspA* is independent of photosynthetic electron transport, while that of *groEL2* is dependent on it (Asadulghani et al. 2003). Thus, induction of heat-shock genes by salt stress may be controlled by a mechanism different from that regulating heat-shock induction. Imamura et al. (2003) have shown reduced heat induction of *hspA* in a *sigB* knockout strain. Diminished salt inducibility was observed in many genes, including *sigB* and heat shock genes such as *hspA* and *dnaK2*, by mutation of *hik34* (Marin et al. 2003). Thus, SigB might be involved in the transduction of the salt signal sensed by Hik34.

The heat shock gene *hspA* is found to confer salt tolerance in the cyanobacterium *Synechocystis* sp. strain PCC 6803. The enhanced expression of GroES and GroEL in the *hspA* mutant, which may compensate for the lack of HspA, may be triggered simply by the absence of HspA, which provides a reservoir for non-native proteins (Török et al. 2001; Kenneth et al. 2004). It is known that the expression of *groEL*s is regulated by HrcA/CIRCE in *Synechocystis* sp. strain PCC 6803 and that, under normal growth conditions, it is repressed by the interaction of HrcA with CIRCE (Nakamoto et al. 2003). A probable molecular mechanism for the relatively high constitutive level of expression of these genes in the *hspA* mutant might include the functional absence of HspA via the continuous accumulation of higher levels of non-native proteins throughout the cell's lifespan. Accumulating non-native proteins may keep GroELs unavailable to interact with HrcA for its

activation and subsequent binding to CIRCE, and thus lead to de-repression of the two *groE* transcripts.

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