Targeted inactivation of the *hrcA* repressor gene in cyanobacteria

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Abstract To determine if the CIRCE/HrcA system operates in cyanobacteria, we have inactivated the *hrcA* repressor gene in *Synechocystis* sp. PCC 6803 by gene targeting. In the *hrcA* mutant, the *groESL1* operon and the *groEL2* gene, both of which have the CIRCE operator in their upstream regions, were derepressed at 30°C without affecting expression of other major heat-shock genes. However, expression of these *groE* genes in the mutant was not fully derepressed. Their transcription increased further upon heat shock, and was initiated from the same sites as those used under normal conditions. This suggests that their expression is regulated by at least two different mechanisms, a negative one controlled by HrcA and an unknown positive one. The heat-induced expression of *clpB1* and *htpG* was greatly repressed by the absence of HrcA. The *hrcA* mutant which constitutively overexpressed GroEL displayed improved cellular thermotolerance and also reduced photobleaching of phycocyanin under heat stress conditions.

Key words: Cyanobacterium; CIRCE; GroEL; Heat-shock protein; HrcA; Phycocyanin

1. Introduction

Cyanobacteria are photoautotrophic prokaryotes that are phylogenetically and physiologically related to the chloroplasts of photosynthetic eukaryotes. Cyanobacteria, like other organisms, synthesize a diverse range of heat-shock proteins (Hsps) upon exposure to high temperatures [1]. In order to demonstrate a specific contribution of an Hsp in thermotolerance in cyanobacteria, disruption mutants of the *clpB*, *hspA*, and *htpG* genes have been constructed [2,3,4]. Interestingly, all of these disruptants showed far more striking thermosensitive phenotypes than those of *Escherichia coli* or other prokaryotes, suggesting that these Hsps have a particularly important role for photosynthetic prokaryotes. Constitutive overexpression of HspA confers thermotolerance and thermal protection of the photosynthetic apparatus in cyanobacteria [5], indicating an important role of a small Hsp in thermal stress management in photoautotrophic organisms.

The regulation of the expression of cyanobacterial heat-shock genes remains poorly understood. So far, two possible *cis*-acting elements have been described in cyanobacteria. One of them is an AT-rich imperfect inverted repeat sequence located in the 5′-untranslated region of *Synechococcus vulcanus hspA* gene [6]. We detected a protein(s) which specifically binds to this sequence. We postulated that the protein may control the transient expression of the *hspA* gene as a repressor, since the DNA-binding activity was transiently lost upon a heat-shock treatment of cells. The other *cis*-acting element is CIRCE (*c*ontrolling *i*nverted *r*epeat of *c*haperone *e*xpression) [7]. Transcriptional start sites of the two heat-inducible *groEL* genes of *Synechocystis* sp. PCC 6803 were mapped within CIRCE and were identical irrespective of the temperature treatment [8]. CIRCE is an inverted repeat consisting of 9 bp separated by a 9-bp spacer; it has been found in more than 40 different eubacterial species including both Gram-positive and Gram-negative species [9], and shown to act as a negative *cis*-element of *grpE*/*dnaK*/*dnaJ* and/or *groE* operons [7,10,11]. *hrcA* (*h*eat shock *r*egulation at *C*IRCE elements) [12] encodes a negative regulator [12–14]. The HrcA repressor protein binds directly to CIRCE [13,15,16]; it thus acts as a repressor and CIRCE as an operator. In *Bacillus subtilis* [7], *Caulobacter crescentus* [12] and *Bradyrhizobium japonicum* [16], the *hrcA* gene is part of the *dnaK* operon and is heat inducible.

Common to all regulatory systems involved in the heat-shock response is the transient induction of heat-shock genes. This implies that, in the case of the CIRCE/HrcA system, HrcA has to be transiently inactivated, assuming that the system plays a major role in the transcriptional control of the genes with CIRCE. In *B. subtilis*, it has been proposed that the activity of HrcA is modulated by the GroEL chaperonin system [15]. According to the model, HrcA, which tends to aggregate, is maintained in an active conformation by GroEL. When stress conditions lead to increased formation of non-native proteins in a cell, GroEL is titrated by these proteins and is less available to activate HrcA than it is under normal conditions. This situation leads to the transcription of the genes. When more than enough GroEL accumulates to chaperone non-native proteins, it inhibits expression of the CIRCE-containing *groE* and *dnaK* operons. This model was challenged by Minder et al. [16] who noted that HrcA of *B. japonicum* was soluble and that DNA binding of the HrcA was not improved by the presence of either the GroE or DnaK chaperone machinery. Instead of GroEL, HrcA from *Bacillus thermoglucosidansius* was refolded and solubilized by DNA [17]. GroEL had only a minor effect on preventing HrcA from aggregating when compared with DNA.

In order to prove that the CIRCE/HrcA system operates in cyanobacteria, we used gene targeting to disrupt an open

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Abbreviations: CIRCE, controlling inverted repeat of chaperone expression; *hrcA*, heat-shock regulation at CIRCE elements; Hsp, heat-shock protein; PCR, polymerase chain reaction

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reading frame (ORF) that encodes a HrcA homologue in *Synechocystis* sp. PCC 6803. As expected, the *groESL1* operon and the *groEL2* gene were derepressed in the mutant under normal conditions, while the CIRCE-lacking heat-shock genes were not. However, either heat shock or irradiation further activated the *groE* genes in the mutant, indicating that another level of control exists. Expression of the *groE* genes was transient in the mutant, indicating that HrcA-reactivation is not the sole mechanism for the feedback inhibition of the *groE* expression in cyanobacteria.

2. Materials and methods

2.1. Organisms and culture conditions

Unless otherwise indicated, *Synechocystis* sp. PCC 6803 cells were grown at 30°C under a light intensity of 30 μE/m^2/s in liquid BG-11 medium [18] 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 HEPES–NaOH (pH 8.0) and 50 μg/ml Na_2CO_3. The liquid culture was bubbled with air.

2.2. Preparation of total RNA

Cells in 15-ml liquid culture that had been incubated for different time intervals after shifting cultures from 30°C to 42°C were immediately collected by centrifugation and frozen in liquid N_2. During the incubation, the culture was illuminated and air-bubbled as described above. Alternatively, cells in 15-ml liquid culture were killed instantaneously by the addition of an equal volume of ice-cold 10% phenol in ethanol, collected by centrifugation, and frozen in liquid N_2 [19]. Both methods gave similar results. Total RNA was extracted as previously described [20].

2.3. Northern blot analysis

Suitable fragments for the preparation of the *clpB1*-, *clpB2*-, *htpG*-, *dnaK2*-, *groEL2*-, *groES*- (to detect transcripts of the *groESL1* bicistronic operon), *hspA*-, and *rnpB*-specific probes for detection of the corresponding RNA from *Synechocystis* sp. PCC 6803 were amplified by polymerase chain reaction (PCR) with genomic DNA as template and appropriate pairs of primers listed in Table 1. PCR products containing part of *htpG*, *dnaK2*, *groEL2*, or *groES* were cloned in either the pT7Blue T-vector (Novagen) or pBluescript II SK (Stratagene), and linearized by digestion with an appropriate restriction enzyme. Digoxigenin-labeled complementary RNA was synthesized *in vitro* by T7 RNA polymerase. For the amplification of DNA fragments containing a portion of either *clpB1, clpB2*, *hspA*, or *rnpB*, an antisense oligonucleotide primer containing either T3 or T7 RNA polymerase promoter was used (Table 1). Thus, the PCR product was directly used for the synthesis of the digoxigenin-labeled RNA probe. After electrophoresis, RNA was blotted onto BM positively charged nylon membrane (Roche), and cross-linked by ultraviolet light irradiation. A separate blot for each particular probe was hybridized at 70°C as instructed by the manufacturer (Roche). After washing the membrane, the chemiluminescence signal from each probe was detected by exposing the blot for different time intervals to X-ray films. The size of mRNA was determined using an RNA ladder (Gibco/BRL).

2.4. Primer extension analysis

Primer extension analysis was performed as described previously [21]. Synthetic oligonucleotides, 5′-TTTCACAGTAGAAACATTAATGGAAATAGC-3′ and 5′-TCTGGATTCATCCTTAAAGGAAATAAG-3′, which are complementary to the region downstream of the transcriptional initiation site of *groESL1* (nucleotides +81 to +110 from the transcription start site) and *groEL2* (nucleotides +54 to +80), respectively, were labeled with [γ-^{32}P]ATP and used as a primer. Primer extension was carried out in 20 μl buffer containing 2 pmol of the labeled primer, 10 μg of total RNA, and 200 units of Superscript™ II RNase H$^-$ reverse transcriptase (Gibco/BRL), and then stopped by the addition of 1 μl of 0.5 M EDTA and 1 μl of RNase A (10 μg/ml). The extended products were electrophoresed in a 6% polyacrylamide, 7 M urea sequencing gel. Products of dideoxynucleotide sequencing reactions performed with the same primer and a cloned *groES*- or *groEL2*-containing DNA fragment as a template were run in parallel to allow determination of the end points of the extension products. The DNA fragments which extend from −299 to +355 (from the transcription start site) of the *groES* gene and from −249 to +1188 of *groEL2* were amplified with the *Synechocystis* genomic DNA as a template and the following primer sets, groE05 (sense) 5′-TCAAGCTCGTGAATGGTGAGAGG-3′ and groE02 (antisense) 5′-GTTAACAGAACATAGTCGTCGC-3′, and cpn-3 (sense) 5′-ATGTCCAAGCTCTAGTGGATGC-3′ and cpn-2 (antisense) 5′-CGACTTTAATCACAGCTACACC-3′, respectively.

2.5. Construction of mutants

A region of the *Synechocystis* sp. PCC 6803 genome containing *hrcA* was amplified by PCR with the P1 and P2 primers (5′-TAGGACGGCCATGGTAAGAT-3′ and 5′-TACTAACCTGTTGCCTGGAG-3′, respectively). The PCR product was cloned in pT7Blue T-vector. The resulting plasmid DNA, pT7Blue-hrcA, contains a unique *Apa*I restriction site in the coding region of the *hrcA* gene. A kanamycin-resistant gene cassette was inserted into the restriction site in the forward direction (the same gene orientation as that of *hrcA*). The cassette was isolated by digesting pUC4K (Pharmacia) with *Pst*I. The construct was used to transform cells of naturally competent *Synechocystis* sp. PCC 6803 through homologous recombination, and these were segregated for a few generations by single colony selection on BG-11 agar plates to isolate mutant strains [22]. Twenty μg/ml kanamycin sulfate was used for selection of transformants. Characteristics of one of the clones are presented.

Table 1
Sequences of oligonucleotide primers used for probe preparation

mRNA to be detected	Primer name	Primer sequence (5′ to 3′)
clpB1	clpB1-3	ACAAAGCTGAGGGGATAATAGC
(slr0156[a])	clpB1-4[b]	AATTAACCCTCACTAAAGGGGTTGTTCTACATAAGCCTGC
clpB2	clpB2-1	CGGAGATTGCTAAACAGCATCG
(slr1641[a])	clpB2-2[b]	AATTAACCCTCACTAAAGGGTCGATTCACTCGGATTAC
htpG	htpG-9	GATTGGTCACTTTGGTTTGGGT
(sll0430[a])	htpG-10	GATATTCTGCCGGATTTTCGTC
dnaK2	dnaK2-1	GCTGCGGAAATTGGTGGACGATG
(sll0170[a])	dnaK2-2	TGGGTGGCAGTGATGAAGGGCA
groES	groES-1	GACCTTGCAAGACTATCATCTG
(slr2075[a])	groES-2	GTTAACAGAACATAGTCGTCGC
groEL2	cpn-1	ATCGGCGTAAAGCTGTGTTGCA
(sll0416[a])	cpn-2	CGACTTTAATCACAGCTACACC
hspA	shsp-1	TTCTTTACAATCCCCTGCGG
(sll1514[a])	shsp-2[c]	TAATACGACTCACTATAGGGTCAAAGTTAGGATACCGGCAT
rnpB	rnaseP-3	AGTTAGGGAGGGAGTTGC
	rnaseP-4[c]	TAATACGACTCACTATAGGGTAAGCCGGGTTCTGTTCC

[a]ORF number assigned in Cyanobase.
[b]The antisense oligonucleotide primer contains the T3 RNA polymerase promoter sequence (the underlined part of the sequence).
[c]The antisense oligonucleotide primer contains the T7 RNA polymerase promoter sequence (the underlined part of the sequence).

2.6. Protein extraction, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and Western blot analysis

Cells growing exponentially were harvested by centrifugation at room temperature and disrupted immediately to extract soluble proteins [6]. Equal amounts of the protein extract 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 [23]. Western blot analysis was performed to detect GroEL and DnaK proteins by using anti-*S. vulcanus* GroEL and DnaK polyclonal antibodies as probes, respectively [23].

2.7. Viability assays

Cells were grown to mid log phase (an absorbance of 0.80 to 1.0 at 730 nm) at 30°C under a light intensity of 30 μE/m^2/s. Twenty ml of the culture were incubated with continuous air-bubbling at 48°C for 15 or 60 min under the same light intensity. Aliquots of the culture taken before and after the high-temperature treatment were diluted to an OD of 0.1 at 730 nm, and then serially diluted in fresh, sterile BG-11 medium. A 10-μl aliquot from each dilution was then spotted onto a BG-11 plate, and the culture was grown at 30°C under a light intensity of 30 μE/m^2/s for 10 days. Cell survival was determined by counting the number of colonies in the most and second most diluted cultures and multiplying their average by the appropriate dilution factor.

3. Results and discussion

3.1. HrcA homologues and CIRCE in cyanobacteria

To date, one ORF encoding a HrcA homologue has been found in each genome of *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120, or *Thermosynechococcus elongatus* BP-1 when searched by Cyanobase (http://www.kazusa.or.jp/cyano/). The deduced primary sequences were aligned with HrcAs from other prokaryotes using Clustal W (data not shown). The similarity among HrcAs from various prokaryotes including cyanobacteria was restricted mainly to three previously described regions, Box A, Box B, and Box C [14].

In contrast to other bacteria, neither *dnaK* nor any other heat-shock genes such as *grpE* and *dnaJ* are located near the *hrcA* genes in *Synechocystis* and other cyanobacteria (Fig. 1A). Although there is a CIRCE element upstream of the *hrcA* genes from bacteria such as *B. subtilis*, *Streptococcus mutans*, and *Chlamydia trachomatis*, all the cyanobacterial *hrcA* genes lack CIRCE. This indicates that the cyanobacterial *hrcA* gene is not subject to autoregulation as it is in *B. subtilis*.

So far, CIRCE has been identified in cyanobacteria around the transcriptional start site of *groE* only, but not near any of the multiple *dnaK* genes. Two distinct *groEL* genes generally exist in a cyanobacterial cell [24–26]. One gene, designated as *groEL1*, is co-transcribed with *groES*, while the other, *groEL2* or *cpn60*, produces a monocistronic mRNA. CIRCE is not present upstream of all the *groE* genes. For example, the *S. vulcanus groEL2* is not associated with CIRCE [25]. We aligned the 5′-upstream regions of *groE* genes from various cyanobacteria (Fig. 1B). The CIRCE sequence, TTAGCACTC-N9-GAGTGCTAA, is perfectly conserved among the *groE* genes. The *groESL1* operons, from *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942, have transcription start sites at the same nucleotide, which is located within CIRCE (Fig. 3A and [27]). The *groESL1* transcription started from the same site under normal and heat-shock conditions. In all the *groESL* operons, the consensus promoter sequences of the *E. coli* major sigma factor were identified just upstream of CIRCE, with the last two nucleotides of the −10

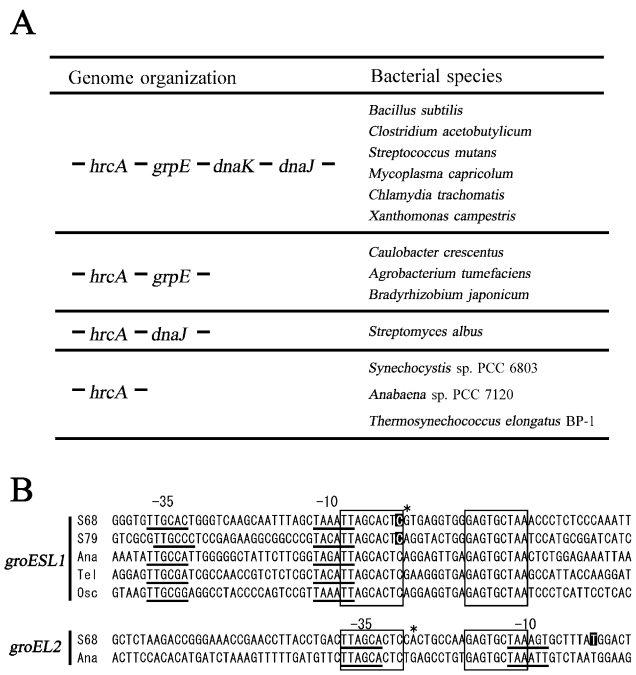

Fig. 1. Genome organization of the *hrcA* gene in various bacteria (A) and alignment of nucleotide sequences of 5′-upstream regions of *groESL1* and *groEL2* from various cyanobacteria (B). In B, the −10 and −35 sequences are underlined, the transcription start sites of *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 *groE* genes that we and Webb et al. [27], respectively, determined are highlighted; the transcription start sites of *Synechocystis* sp. PCC 6803 *groE* genes that Glatz et al. [8] determined are indicated by asterisks, and the inverted repeat of CIRCE is boxed. S68, *Synechocystis* sp. PCC 6803; S79, *Synechococcus* sp. PCC 7942; Ana, *Anabaena* sp. PCC 7120; Tel, *T. elongatus* BP-1; Osc, *Oscillatoria* sp. NKBG 091600. The *groE* nucleotide sequences of Osc and S79 were taken from GenBank, and the other sequences were from Cyanobase.

sequence overlapping with the first two nucleotides of CIRCE. Thus it is likely that the promoter for the *groESL* operon is recognized by the major sigma factor in cyanobacteria as in the case of other prokaryotes such as *B. subtilis*. We could not find any promoter sequences upstream of the transcription start site of the *groEL2* gene from *Synechocystis* sp. PCC 6803 that was identified by Glatz et al. [8] (Fig. 1B, see the nucleotide indicated by an asterisk). As shown in Fig. 3B, we found a different transcriptional start site for the *groEL2* gene (the highlighted nucleotide), upstream of which the consensus promoter sequence was found. Most of the *groEL2* promoter overlaps with CIRCE. We also found consensus promoter sequences that overlap with CIRCE upstream of the *Anabaena groEL2* gene by a computer search for promoters (Fig. 1B).

3.2. Disruption of hrcA

To analyze the role of HrcA in heat-shock gene regulation of *Synechocystis* sp. PCC 6803, an ORF, sll1670, was disrupted by double crossover insertion of a kanamycin resistance gene cassette into the chromosome. Correct integration and complete segregation of the mutation in all the copies of the genome was confirmed by PCR. These results indicate that the gene is not essential under normal growth conditions. No detectable difference in growth rates was observed at 30°C (data not shown). The mutant cells grew similarly to the wild type during the exponential growth phase at 42°C, but

slightly more cells accumulated than for the wild type after reaching the stationary phase. This may be indicative of a growth advantage for the mutant at a moderately high temperature.

3.3. *Derepression of the* groESL1 *and* groEL2 *genes in the* hrcA *disruptant under normal growth conditions*

Consistent with the hypothesis that sll1670 encodes the HrcA repressor, the inactivation of the ORF led to derepression of *groESL1* and *groEL2* under normal growth conditions (Fig. 2A, 0 min; see also Fig. 3). The level of the GroEL protein was significantly elevated in the mutant grown under normal growth conditions (Fig. 2B, 0 min), confirming the results obtained by RNA analysis and further strengthening our conclusion that *hrcA* encodes a negative regulator of the cyanobacterial *groE* genes.

However, disruption of *hrcA* had no marked effect at 30°C on expression of *clpB1, clpB2, htpG*, *dnaK2*, or *hspA* (Fig. 2A, 0 min). With the exception of *clpB2*, these genes are not transcribed significantly under normal conditions. This is in contrast to the constitutive *rnpB* RNA expression that was analyzed as a reference for the detection of heat induction. The level of DnaK did not change as a consequence of the lack of HrcA (Fig. 2B, 0 min). The upstream regions for these genes lack CIRCE element consensus sequences. Thus, the CIRCE/HrcA system appears to be specific to the *groE* genes, although we cannot rule out the possibility that other genes, not tested in the assay above, may also be regulated by this system.

Previously, there was no transcriptional analysis on the heat induction of cyanobacterial *clpB*s. In the *Synechocystis* genome, there are two ORFs encoding ClpB homologues that are found in Cyanobase. In the present study, we examined transcription of both *clpB1* and *clpB2*. As shown in Fig. 2A with the wild type cells, expression of *clpB1* is dependent on heat shock, while that of *clpB2* is constitutive. Thus, there is no possibility that the *clpB1* probe also detects transcripts of *clpB2* or vice versa, although the sizes for the two *clpB* mRNAs are similar. The induction patterns for the two *clpB* genes from *Synechocystis* are similar to those for the ClpB proteins from *Synechococcus*. The ClpB1 protein in *Synechococcus* sp. PCC 7942 is induced by heat shock, while the ClpB2 protein is a constitutive protein [28].

3.4. *Existence of another level of heat-shock control for the* groESL1 *and* groEL2 *genes*

The levels of *groESL1* and *groEL2* mRNAs (Fig. 2A) and the GroEL protein level (Fig. 2B) in the mutant cells were increased further by a heat-shock treatment at 42°C. Thus, the expression of the two *groE* genes was not fully derepressed at 30°C by disruption of *hrcA*, suggesting that another level of heat-shock control exists. This control is likely to play a major role in *groE* heat induction because of the great increase in the level of *groE* mRNA upon heat shock in the *hrcA* mutant.

In order to precisely identify the transcriptional start sites from which derepressed and heat-induced transcriptions of both the *groESL1* operon and the *groEL2* gene took place, primer extension analysis with RNA from non-heat-shocked and heat-shocked *Synechocystis* cells was performed. Single transcriptional start sites for the *groESL* operon and the *groEL2* gene were found 75 bp and 44 bp upstream, respectively, of the translational start codons in the wild type cells

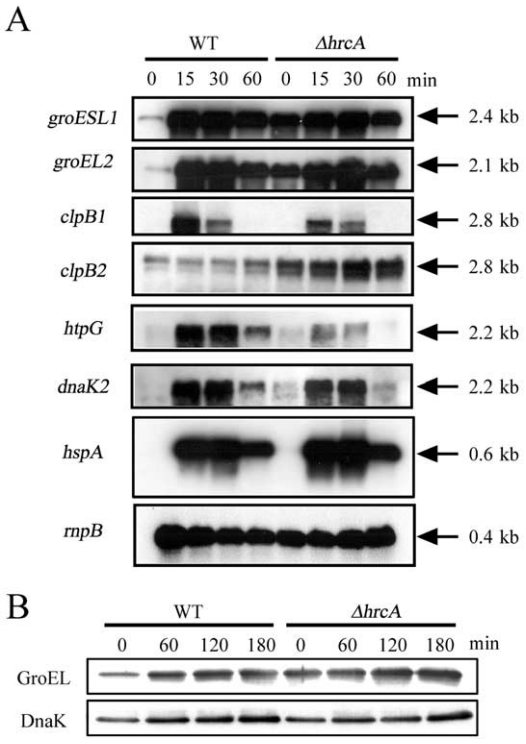

Fig. 2. Northern and Western blot analyses to show the heat-induced accumulation of transcription and translation products of various heat-shock genes from the wild type (WT) and *hrcA* mutant (*ΔhrcA*) strains of *Synechocystis* sp. PCC 6803 after a shift to 42°C in the presence of light (30 μE/m^2/s). A: Time course for the accumulation of mRNAs of various heat-shock genes. Cells in a 70-ml culture growing at 30°C under a light intensity of 30 μE/m^2/s were subjected to heat shock and 15-ml aliquots of the culture were collected at 15-, 30-, and 60-min intervals. 0.6 μg (for the *groESL1*, *groEL2*, and *hspA* mRNA detection), 0.6 μg (for the *rnpB* RNA detection), 1.7 μg (for the *clpB1* and *clpB2* mRNA detection), 2.0 μg (for the *htpG* mRNA detection), or 3.6 μg (for the *dnaK2* mRNA detection) of total RNA were used for Northern blotting. B: Time course for the accumulation of the GroEL and DnaK proteins. Cells in a 70-ml culture growing at 30°C under a light intensity of 30 μE/m^2/s were subjected to heat shock and 15-ml aliquots of the culture were collected at 60-, 120-, and 180-min intervals. Either 10 μg (for the GroEL detection) or 15 μg (for the DnaK detection) of crude soluble proteins were separated by SDS–PAGE (16% acrylamide gel), and then GroEL and DnaK were specifically detected by Western blot analysis using polyclonal antibodies raised against *S. vulcanus* GroEL and DnaK.

(Fig. 3A,B). Glatz et al. [8] also found single transcriptional start sites for *groESL1* and *groEL2*, but theirs are different from the start sites we determined. The one for the *groESL1* operon and *groEL2* were located one base downstream and 27 bp upstream, respectively, of ours. Consensus promoter sequences are found upstream of the transcriptional start sites for *groESL1* and *groEL2* as determined here. The transcription of both *groESL1* and *groEL2* from the vegetative promoters overlapping CIRCE was clearly derepressed at 30°C in the *hrcA* mutant (Fig. 3). Thus, our results strongly indicate that the transcription of *groEL1 and groEL2* is repressed under normal growth conditions by the interaction of HrcA with CIRCE. As proposed in *Bacillus*, the *groE* genes in the wild type may be heat-induced (or derepressed) due to the inactivation of the HrcA protein under heat stress. However, there may be other mechanisms which control the cyanobacterial *groE* gene expression, as discussed above. The heat-in-

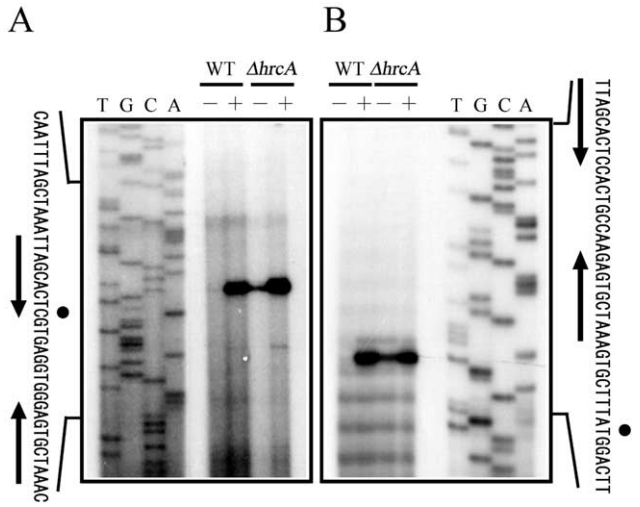

Fig. 3. Mapping of the 5′-end of the *groESL1* (A) and *groEL2* (B) mRNAs by primer extension analysis. Primer extension reactions were performed with total RNA isolated from the wild type cells (WT) or the *hrcA* mutant (*ΔhrcA*) before (−) and after (+) shifting from 30 to 42°C for 30 min in the light (30 μE/m^2/s). T, G, C, and A indicate the dideoxy sequencing ladder obtained with the same oligonucleotide as that used for the primer extension analysis and a cloned genomic DNA containing either *groES* or *groEL2* as a template. The nucleotide sequence containing CIRCE is shown. Paired inverted arrows indicate the location of the consensus CIRCE inverted repeat sequences. The potential transcription start site is marked with a black circle.

duced transcription in the *hrcA* mutant took place from the same start site as the one under normal conditions. A possible interpretation of the results is that heat stress activates transcription of the two *groE* genes by the RNA polymerase with the major sigma factor.

The presence of multiple regulatory mechanisms for *groE* gene expression is further supported by the fact that the transcriptions of the *groESL1* operon and the *groEL2* gene in the *hrcA* mutant as well as the wild type are light-activated at a normal growth temperature (data not shown). The increase in the level of the *groE* transcripts upon a shift of cells from dark to light took place independent of HrcA.

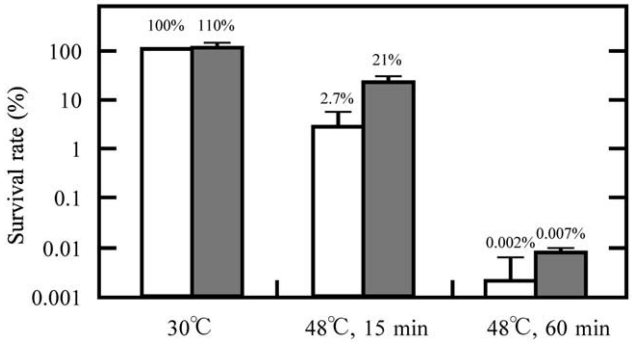

Fig. 4. Survival rate of the wild type (open column) and the *hrcA* mutant (shaded column) after a direct shift to 48°C for 15 or 60 min in the light (30 μE/m^2/s). For all treatments, the number of the wild type cells present before the shift was taken as 100%. Each value shows the mean and standard deviation of four independent replicate experiments.

3.5. *Effect of the disruption of* hrcA *on the heat-induced expression of other major heat-shock genes*

Disruption of *hrcA* led to a strong inhibition of *htpG* and *clpB1* heat induction, while it did not have any effect on *hspA* induction (Fig. 2A). The mRNA accumulation of *dnaK2* was slightly reduced in the mutant. The heat-induced accumulation of the DnaK protein appeared to be slightly slower in the mutant than the wild type. A likely interpretation of these results is that the enhanced cellular level of GroES and GroEL before heat shock represses the heat inductions of *clpB1*, *htpG*, and *dnaK2*. Interestingly, the *clpB2* gene, whose mRNA level was constant in the wild type irrespective of the temperature treatment, was heat-induced in the *hrcA* mutant. This increase in the *clpB2* expression may complement the decrease in the *clpB1* heat induction in the mutant, although nothing is known about the underlying mechanism.

3.6. *No major involvement of HrcA with the shut-off of the* groE *transcription*

As described in the Introduction, HrcA has been proposed to be involved in the shut-off of heat induction of the *groE* and *dnaK* operons in *B. subtilis* [15]. In *Synechocystis*, how-

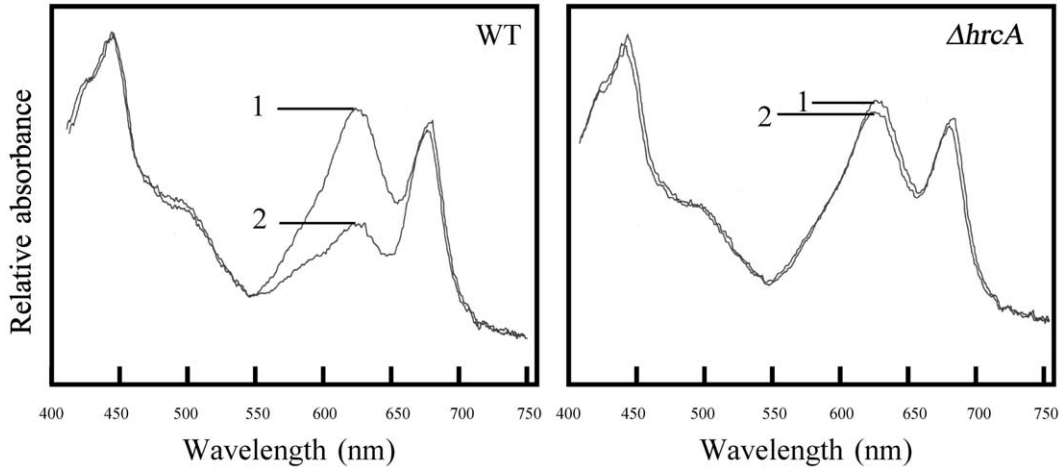

Fig. 5. Absorption spectra of the wild type and the *hrcA* mutant (*ΔhrcA*) cells in a 50-ml culture grown at 30°C either before (1) or after (2) a shift to 50°C for 10 min in the light (30 μE/m^2/s). Whole cell absorbance was measured at room temperature with a Hitachi 557 dual wavelength double beam spectrophotometer (Hitachi Koki, Tokyo, Japan).

ever, the absence of HrcA did not affect the transient accumulation of the *groE* mRNAs (Fig. 2A). We confirmed the transient accumulation of *groESL1* and *groEL2* transcripts in the *hrcA* mutant as well as the wild type by measuring their mRNA levels up to 18 h (data not shown). After 18 h incubation at 42°C, these transcripts in both the wild type and the *hrcA* mutant cells were reduced to a negligible level, although the time course of the mRNA level showed that the level decreases more slowly in the *hrcA* mutant than the wild type. These results indicate that HrcA does not play a major role in the transient accumulation of the *groE* mRNA in cyanobacteria. Shut-off of *groE* induction in the absence of the HrcA repressor was also observed in *B. subtilis* [29]. As postulated by Homuth et al. [30], posttranscriptional regulation affecting mRNA stability of CIRCE containing transcripts might be involved in the transient accumulation of the *groE* mRNAs.

3.7. *Effect of the disruption of* hrcA *on the cellular thermotolerance and the thermal protection of the photosynthetic apparatus*

We tested whether the *hrcA* disruption would affect the performance of *Synechocystis* sp. PCC 6803 under heat stress conditions. We analyzed the mutant's ability to survive a temperature upshift from the growth temperature of 30°C to 48°C, which is lethal to the wild type *Synechocystis*. As shown in Fig. 4, the *hrcA* disruption resulted in eight-fold and four-fold increases in the survival rate after the high-temperature treatment for 15 min and 60 min, respectively. In contrast to our present results, the *hrcA* deletion did not improve the heat tolerance of *C. crescentus* [12] or *B. japonicum* [16]. On the other hand, the *hrcA* mutant of *B. subtilis* acquired thermotolerance [13]. Minder et al. [16] suggested a possible reason for the discrepancy between *B. subtilis, C. crescentus,* and *B. japonicum* lies in the fact that *C. crescentus,* and *B. japonicum hrcA* mutants enhance only the concentration of GroE chaperonins, whereas the lack of HrcA in *B. subtilis* leads to an overexpression of both GroESL and DnaKJ/GrpE. Only increased level of the complete set of major chaperones would improve the capacity of bacterial cells to survive temperature upshift. However, in cyanobacteria, although the DnaK level is not changed under normal growth conditions, the *hrcA* disruption conferred the basal thermotolerance to cyanobacteria.

Previously, we showed that phycocyanin is photo-bleached by a short heat-shock treatment of *Synechococcus* sp. PCC 7942 under weak light [5]. This photo-bleaching was greatly suppressed by a constitutive over-production of HspA in the cyanobacterium, indicating that the small Hsp can protect the light absorbing ability of phycocyanin under stress conditions. Similar to the small Hsp-overexpressing *Synechococcus* sp. PCC 7942, the *Synechocystis hrcA* mutant was much more resistant than the wild type to the heat-induced photo-bleaching of phycocyanin (Fig. 5). A possible interpretation of these results is that GroE as well as small Hsp can protect phycocyanin from thermal photo-bleaching. We propose that different molecular chaperones may cooperate in the protection of phycocyanin and the phycobilisome complex.

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