RNA helicase, CrhR, regulates the low temperature-inducible expression of heat shock genes *groES*, *groEL1* and *groEL2* in *Synechocystis* sp. PCC 6803

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Running title: RNA helicase and expression of heat shock genes

Abstract The *crhR* gene for RNA helicase, CrhR, was one of the most highly induced genes, when the cyanobacterium *Synechocystis* sp. PCC 6803 was exposed to a downward shift in ambient temperature. Although CrhR may be involved in the acclimatization of cyanobacterial cells to low-temperature environments, its functional role during the acclimatization is not known. In the present study, we mutated the *crhR* gene by replacement with a spectinomycin resistance gene cassette. ResultantΔ *crhR* mutant cells exhibited a phenotype of slow growth at low temperatures. DNA microarray analysis of the genome-wide expression of genes, Northern and Western blotting analyses indicated that mutation of the *crhR* gene repressed the low temperature-inducible expression of heat-shock genes, *groEL1* and *groEL2*, at the transcript and protein levels. The kinetics of the *groESL* co-transcript and the *groEL2* transcript after addition of rifampicin suggested that CrhR stabilized these transcripts at an early phase, namely, 5-60 min, during acclimatization to low temperatures and enhanced the transcription of these genes at a later time, namely, 3-5 h. Our results suggest that CrhR regulates the low temperature-inducible expression of these heat-shock proteins, which, in turn, may be essential for acclimatization of *Synechocystis* cells to low temperatures.

Key words: Chaperonin, Cyanobacterium, *groEL*, Low-temperature stress, RNA helicase, *Synechocystis*

INTRODUCTION

Low-temperature stress is one of the most important factors of environment that limit various biological activities. Organisms perceive the low-temperature stress and regulate the expression of various genes whose products are supposed to be important for the acclimatization to low-temperature environments (Polissi *et al.*, 2003).

We have been working on the mechanisms of acclimatization to low-temperature stress of the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*). Studies using DNA microarrays demonstrated that low-temperature stress induced the expression of a large number of genes that include genes for ribosomal proteins, RNA binding proteins, subunits of RNA polymerases, subunits of NADH dehydrogenase, acyl-lipid desaturases, and proteins of as yet unknown function (Suzuki *et al*., 2001; Los & Murata, 2002; Murata & Los, 2006). However, the functional role of these proteins during acclimatization to low temperature is not known, except for acyl-lipid desaturases, which are supposed to optimize the fluidity in membranes at low temperatures (Los *et al.*, 2008).

The *crhR* gene for RNA helicase is one of such genes whose expression is highly induced upon low-temperature stress in *Synechocystis* (Suzuki *et al.*, 2001; Los *et al.*, 2008). The database of genome sequences available at http://www.kazusa.or.jp suggests that *Synechocystis* contains only one gene, *crhR* (open reading frame, *slr0083*), for RNA helicase. Heterologous expression of the *crhR* gene in *E. coli* and subsequent biochemical characterization of the expressed protein demonstrated that this protein

68 catalyzed both ATP-dependent unwinding of secondary structures of RNA and annealing
69 of complementary RNA strands (Chamot *et al.,* 2005). Although CrhR *in vitro* is active
70 in unwinding, annealing and exchanging RNA strands, its function during acclimatization
71 of *Synechocystis* cells to low temperature has not been demonstrated.
72
73 RNA helicases are ubiguitously distributed in all the biological kingdoms (Rocak &
74 Linder, 2004). Extensive studies have demonstrated that they are active in modulating
75 the secondary structure of RNAs by unwinding RNAs in an ATP-dependent manner
76 (Tanner & Linder, 2001). They participate in various cellular processes in which RNAs
77 are involved. In model experimental organisms, such as *E. coli,* a cold-inducible RNA
78 helicase, CsdA, has been suggested to participate in the assembly of ribosomes (Lauri *et*
79 *al.,* 2008). Another RNA helicase is a component of 'cold shock degradosome complex'
80 and enhances the degradation of mRNAs by unwinding the secondary structures so as to
81 facilitate cleavage of mRNA by RNase E (Carpousis *et al.,* 1999; Prud'homme-Genereux
82 *et al.,* 2004).
83
84 The cyanobacterium *Anabaena* sp. PCC 7120 contains two genes for RNA helicases; the
85 *crhC* gene is induced only under low-temperature stress whereas the *crhB* gene is induced
86 by salt, low-temperature and light stress, as well as by nitrogen limitation (Chamot *et al.,*
87 1999). Biochemical characterization and cellular localization of CrhC suggested that it is
88 a membrane-bound protein and may be involved in translocation of proteins across the
89 plasma membrane under low-temperature conditions (El-Fahmawi & Owttrim, 2003).
90

In this work, we intended to elucidate the functional role of RNA helicase, CrhR, during acclimatization of *Synechocystis* cells to low temperatures by investigating changes in the genome-wide expression of genes. We demonstrated that the most important change in the gene expression by mutation of the *crhR* gene appeared in low-temperature inducibility of heat-shock genes, *groESL* and *groEL2*, in both transcript and protein levels; namely, the *crhR* mutation converted these genes from low temperature-inducible to low temperature-non-inducible.

METHODS

Cells and culture conditions. A strain of *Synechocystis*, which is tolerant to glucose (Williams, 1988), was originally obtained from Dr. J. G. K. Williams (Dupont de Nemours). Wild-type cells were grown at 34^{o}C in BG-11 medium (Stanier *et al.*, 1971), which had been buffered with 20 mM HEPES-NaOH (pH 7.5), under continuous illumination from incandescent lamps, as described previously (Wada & Murata, 1989). Δ*crh*R mutant cells (see below), in which the *crhR* gene had been replaced by the spectinomycin resistance gene (*Sp*^{r}) cassette in the genome, were grown under the same conditions as described above with the exception that the culture medium contained spectinomycin at 25 μg ml^{-1} during the pre-cultures. Δ*crhR* mutant cells were then transferred to the above-mentioned BG11 medium, which did not contain spectinomycin, for the final cell culture for the experiments.

Deletional mutagenesis of the *crhR* gene for RNA helicase to generate Δ*crhR* mutant cells. We generated a Δ*crhR* mutant by replacing the *crhR* gene (*slr0083*) by the *sp*^{r} cassette (Fig. 1S): A 767-bp upstream and 960-bp downstream flanking regions of open reading frame *slr0083* from the genomic DNA were amplified using primer sets UF (5'AAT CTA GAG TCG ATA TTC CTT GGA TTC GTA TT 3') and UR (5'AAA GGC CTG ACG GTT TAG TGG GCA AAT AAT T 3'); DF (5'AAA GGC CT AAC TCC TCC AGA ACT AAG ACC 3') and DR (5'AAG AGC TCC ATC GAA CCC ATT GAC CTA GAG 3'), respectively. An *Xba*I site and a *Sac*I site (underlined) were created in UF and DR primers, respectively, during primer synthesis. A *Stu*I site (underlined) was created in the primers UR and DF. The PCR fragments, thus generated using UF-UR and DF-DR primer sets, were cloned onto pT7Blue T-A cloning vector separately and named as pTcrh767 and pTcrh960, respectively. The DF-DR fragment released from pTcrh960 plasmid after digestion with *Stu*I and *Sac*I restriction enzymes was ligated to the same sites on pTcrh767. The resultant construct was named pTcrh$^-$. The *Dra*I digested Omega *Sp*^{r} cassette was cloned onto the *Stu*I site of pTcrh$^-$ construct by the blunt end ligation. Thus, the final construct, in which the *slr0083* open-reading frame had been replaced by the Omega *Sp*^{r} cassette, was used to transform wild-type cells of *Synechocystis*. Genomic DNA extracted from mutant cells was used as the template and UF and DR were used as primers to examine, by PCR, the extent of replacement of the wild-type copy of the chromosome by the mutated copy of the chromosome (Fig. 1Sb). This analysis indicated that the wild-type copy of the *crhR* gene had been completely replaced by the mutated copy in Δ*crhR* cells. The resultant mutant was designated as Δ*crhR*.

Complementation of Δ*crhR* mutation. We complemented Δ*crhR* cells with an autonomous replication plasmid (pVZ321) of cyanobacteria, which harbored the *crhR* gene: A DNA fragment that included the *crhR* gene and its upstream of 400 bp was amplified, by PCR, with the genomic DNA from wild-type *Synechocystis* cells, with the forward primer 5'-GCC CAA AGC TTT GCC CGA AGA AGT AAT G-3' and the reverse primer 5'-CCG TTC TCG AGG AGT TAT TTT TTT CCG AGT C-3'. A *Hind*III and an *Xho*I sites (underlined) were created in forward and reverse primers, respectively. The amplified product by PCR was digested with *Hind*III and *Xho*I and the resultant fragment of 2,007 bp was inserted into a cyanobacterial autonomous replication plasmid, pVZ321 (Zinchenko *et al.,* 1999), which had been digested with the same enzymes. The resultant plasmid was introduced into Δ *crhR* cells by triparental gene transfer (Zinchenko *et al.,* 1999). The complemented strain was designated as *crhR*$^{+}$.

Preparation of cDNAs for DNA microarray analysis. *Synechocystis* cells that had been exposed to low-temperature stress were killed instantaneously by the addition of 50 ml of a mixture of ice-cold phenol/ethanol (1:20 w/v) to 50 ml of the cell suspension and then total RNA was extracted as described previously (Los *et al.,* 1997). The RNA was treated with DNase I (Nippon Gene, Tokyo, Japan) to remove contaminating DNA. cDNAs, labeled with fluorescent dyes (Cy3 and Cy5; Amersham Pharmacia Biotech), were prepared from 10 μg of total RNA with an RNA Fluorescence Labeling Core kit (M-MLV, version 2.0; Takara Co. Ltd., Kyoto, Japan) according to the manufacturer's instructions.

DNA microarray analysis. Genome-wide analysis of transcript levels was performed with DNA microarrays, as described previously (Kanesaki *et al.*, 2002). In brief, we used the *Synechocystis* DNA microarray (CyanoCHIP, Takara Co. Ltd.) that covered 3079 of the 3168 open-reading frames (97% of total genes except transposon-related genes) of the *Synechocystis* genome. Hybridization of the labeled cDNA to DNA microarray was carried out at 65°C for 16 h. After hybridization, the microarrays were rinsed with 2× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate) at room temperature. They were washed with 2× SSC at 60°C for 10 min and 0.2× SSC, 0.1% SDS at 60°C for 10 min and then rinsed with distilled water at room temperature for 2 min. Moisture was removed with the air spray prior to analysis with the array scanner (GMS418; Affimetrix, Woburn, MA). Each signal was quantified with the ImaGene ver. 4.0 program (BioDiscovery, Los Angeles, CA, USA). The signal from each gene on the microarray was normalized with reference to the total intensity of signals from all genes with the exception of genes for rRNAs. Then we calculated changes in the level of the mRNA of each gene relative to the total level of mRNA.

Northern blotting analysis. Total RNA was extracted from cells, and Northern blotting analysis was performed as described previously (Los *et al.*, 1997). In case of mRNA stability experiments, wild-type and Δ*crhR* cells were incubated in the presence of a transcriptional inhibitor, rifampicin, at a final concentration of 100 μg ml^{-1} to determine the stability of *groESL* co-transcript and *groEL2* transcript. At various time points after addition of rifampicin, cells were harvested for RNA extraction and subsequent Northern

blotting analysis. DNA fragments corresponding to *groESL*, *groEL2* and *rnpB* mRNAs were conjugated with alkaline phosphatase (Alkphos Direct kit; Amersham Pharmacia Biotech, Uppsala, Sweden) and the resultant conjugates were used as probes. After hybridization, the blots were soaked with the CDP-star solution (Amersham Pharmacia Biotech) and signals from hybridized mRNAs were detected with a luminescence image analyzer (LAS-1000; Fuji-Photo Film, Tokyo, Japan).

Preparation of antibodies against CrhR. Antibodies against CrhR were raised in rats with His-tagged CrhR of *Synechocystis*, which had been overexpressed in *E. coli*. First, the *crhR* ORF was amplified, by PCR, with the forward primer 5'-GCCATATGACTAATACTTTGACTAGTAC-3' and the reverse primer 5'-GCGTCGACTTACTGTTGGCGATCACTATAG-3' and purified by electrophoresis on agarose gel. The amplified ORF of *crhR* was eluted from the gel and was inserted into pET-28a(+) at *Nde*I and *Sal*I sites to generate pET-CrhR. The N-terminal His-tagged CrhR protein was expressed in BL21(DE3)pLysS, which had been transformed with pET-CrhR, and was purified using HIS-Select^{TM} Nickel Affinity gel (SIGMA - P6611) according to the supplier's instruction. The expression of CrhR protein was induced by addition of 400 mM final concentration of isopropyl β-D-1-thiogalactopyranoside (IPTG). Bacterial cells were collected by centrifugation at 10,000 x g for 10 min and pelletted cells were disrupted with a sonic oscillator **(**Model: UV2070, Probe: MS-72, Bandelin Electronic, Berlin, Germany) operated for 10 min at 50% power, with one min pulse interval, in 100 mM Tris-HCl (pH 8.0) and 200 mM NaCl. Insoluble materials (precipitates) were removed by centrifugation at 20,000 x *g* for 20 min at 4^{o}C. The

supernatant was loaded onto a HIS-Select Nickel Affinity column. After the column was washed with 100 mM Tris-HCl (pH 8.0), 200 mM NaCl and 10 mM imidazole and sequentially with the same buffer that contained 40 mM imidazole. Then, His-tagged CrhR was eluted with 100 mM Tris-HCl (pH 8.0), 200 mM NaCl and 200 mM imidazole. The purity of each fraction was examined by SDS-gel electrophoresis. The fractions, which gave a single band at the expected region on the gel, were combined and dialysed against 5 mM Tris-HCl (pH 8.0). The resultant protein was used to generate anti-CrhR antibodies in rats (by TakaraBio Co. Ltd., Ohtu, Japan).

Western blotting analysis. Soluble proteins were extracted from *Synechocystis* cells by mechanical disruption of cells using glass beads (106 μm in diameter; Sigma Co. Ltd.). *Synechocystis* cells in 200 μl of 50 mM Tris-HCl (pH 8.0) buffer were mixed with 330 mg glass beads in a thick-walled glass tube and disrupted by vigorous vortexing at maximum speed on a vortex mixer for 1 min followed by 2 min cooling on ice. Vortex mixing and cooling on ice was repeated 10 times to make sure maximum disruption of cells. Then, mechanically disrupted cells were centrifuged at 25,000 x g for 20 min at 4^{o}C to separate soluble proteins from insoluble materials. The resultant supernatants, which contained soluble proteins from control and treated cells, were loaded onto SDS-PAGE on equal protein basis (25 μg soluble protein in each well). After electrophoresis, the separated proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore Inc., Billerica, MA, USA) in a semidry transfer apparatus (Atto Co., Tokyo, Japan). Levels of CrhR and GroEL were determined immunologically with an enhanced chemiluminescence Western-blotting kit with specific antibodies against CrhR and

GroEL according to the protocol supplied with the kit (Amersham International, Buckinghamshire, UK). To investigate the level of CrhR, we used horseradish peroxidase-linked antibody raised in goat against rat immunoglobulin G as the secondary antibody. To investigate the level of GroEL1 and GroEL2, we used antibodies raised in rabbits against *E. coli* GroEL and purchased from Sigma Inc. (Catalog No: G-6532; St. Louis, MO, USA**)** as the primary antibody and horseradish peroxidase antibody raised in donkey against rabbit Immunoglobulin G as the secondary antibody. Luminescence image analyzer (LAS-1000; Fuji-Photo Film, Tokyo, Japan) was used to monitor signals from blotted membranes.

RESULTS AND DISCUSSION

The expression of the *crhR* gene was induced upon a downward shift in temperature

The expression of the *crhR* gene is induced under low-temperature, salt and hyperosmotic stress in *Synechocystis* (Vinnemeier & Hagemann, 1999; Suzuki *et al.,* 2001; Kanesaki *et al.,* 2002). Fig. 1a shows changes in the level of *crhR* mRNA during exposure of *Synechocystis* cells, which had been grown at 34 ^{o}C, to 24 ^{o}C for designated periods of time. A low level of *crhR* mRNA was detected before the exposure, indicating that this gene was constitutively expressed in *Synechocystis.* The downward shift in temperature increased transiently the level of *crhR* transcript with the maximum attained at 30 min during the incubation at 24 ^{o}C (Figs. 1a, 1c).

Induction of CrhR after a downward shift in temperature

To examine how the level of CrhR protein reflects the transient increase in the level of *crhR* mRNA during incubation at low temperature, we performed Western blotting analysis of changes in the CrhR level during exposure to 24 ^{o}C of *Synechocystis* cells, which had been grown at 34 ^{o}C for 16 h (Figs. 1b, 1c). The anti-CrhR antibody raised in rats detected CrhR at a molecular mass of approximately 52 kDa (Fig. 1b). Fig. 1c shows that CrhR was present at a certain level before the downward shift in temperature. Then the level of CrhR gradually increased during incubation at 24 ^{o}C and reached a maximum level that was 3.5 times as high as the original after incubation at 24 ^{o}C for 180 min (Fig. 1c). These observations indicated that *Synechocystis* cells induced the expression of the *crhR* gene for RNA helicase and accumulated CrhR after the temperature was shifted downward by 10 ^{o}C from growth conditions. Our previous study with DNA microarray demonstrated that almost all of the low temperature-inducible genes in *Synechocystis* are under the control of the sensory kinase, Hik33, except the *crhR* gene for RNA helicase, (Suzuki *et al.*, 2001). This indicates that the low temperature-inducible expression of the *crhR* gene might be regulated by a mechanism other than the two-component signal transduction.

Mutation of the *crhR* gene and complementation of the Δ*crhR* mutant

In order to confirm that the changes in phenotype due to the mutation of the *crhR* gene, as will be discussed in the next sections, were caused by mutation of the *crhR* gene, we

compared the growth profile of wild-type, Δ*crhR* mutant, and *crhR*-complemented *crhR*$^+$ cells at 24 ^{o}C and 34 ^{o}C. At 34 ^{o}C these three types of cell revealed similar profiles of growth (Fig. 2a). At 24 ^{o}C, however, growth of Δ*crhR* cells was much slower than that of wild-type cells (Fig. 2b), indicating that the low temperature-induced expression of the *crhR* gene and synthesis of CrhR were important for *Synechocystis* cells to grow at low temperature. Complementation of Δ*crhR* cells *in trans* with a functional CrhR expressed from pVZ-p*crhR* (*crhR*$^+$ strain) recovered the growth at the low temperature, which was similar to that of wild-type cells (Fig. 2b). The functional complementation of Δ*crhR* cells by the *crhR* gene clearly demonstrated that CrhR plays an important role in the proper physiology of *Synechocystis* cells at low temperatures.

Genes whose expression at low temperature was affected by mutation of the *crhR* gene

E. coli has two genes for RNA helicases. CsdA is a low temperature-inducible RNA helicase that resumes the translation by unwinding the base-paired regions of mRNA, which are formed at low temperatures (Jones *et al*., 1996). Another RNA helicase, RhlB, is a component of the degradosome complex and is involved in controlling the level of mRNAs (Carpousis *et al*., 1999). Therefore, it seems likely that the mutation of the *crhR* gene in *Synechocystis* would result in changes in gene expression at the transcript level. To examine genes whose expression by low temperature is regulated by CrhR, we analyzed the genome-wide expression of genes in Δ *crhR* mutant cells by DNA microarray method.

First, we investigated the effect of *crhR* mutation on the genome-wide expression of genes under normal growth conditions. Our previous study with the DNA microarray, using RNA extracted from wild-type cells of *Synechocystis* that had been grown under optimal growth conditions (namely, growth temperature, 34 ^{o}C; light intensity, 70 µmol photons m^2 s^{-1}; and CO_2 concentration, 1%) indicated that most of the genes appeared at induction factors between 2.0 and 0.5. These genes are assigned as those whose expression was not significantly affected by low temperature or mutation. The data points that appeared above the induction factor 2.0 or below the induction factor 0.5 represent those genes whose expression was induced or repressed, respectively, due to the mutation or by stress conditions (Suzuki *et al.*, 2001; Kanesaki *et al.*, 2002). Microarray analysis indicated that the mutation in Δ *crhR* cells had no significant effect on the genome-wide expression of genes under isothermal conditions at 34^{o}C (Fig. 2S).

Next, we performed DNA microarray analysis to investigate whether the mutation in Δ*crhR* cells would have any effect on the low temperature-induced expression of genes. Figs. 3Sa, 3Sb and 3Sc represent the gene expression profiles in wild-type cells after exposure of respective cells to low temperature for 20, 60 and 180 min, respectively. Figs. 3Sd, 3Se and 3Sf represent the gene expression profiles in Δ*crhR* cells under similar conditions. The inducibility by low temperature of the majority of genes was not much affected by the mutation of *crhR*. Only a very small number of genes were significantly affected by the mutation in Δ *crhR*. The genes for molecular chaperonins, *groES*, *groEL* and *groEL2*, are indicated in closed circled data points in the scatter plots in Fig. 3S.

Table 1 lists genes whose low-temperature inducibility was significantly affected by mutation of the *crhR* gene. The mutation significantly enhanced the low temperature-inducible expression of genes *pyrB, gifA, gifB* and *slr0082*, whereas it decreased that of *groES, groEL1, groEL2* and *sll1611*. Although the *slr0082* gene for a hypothetical protein, which is located upstream of the *crhR* (*slr0083*) gene in the *Synechocystis* genome, and the *crhR* gene are transcribed by their own promoters (Vinnemeier & Hagemann, 1999), the mutation in the *crhR* gene enhanced the expression of *slr0082* (Table 1). These observations might suggest that there was a regulatory mechanism, which interacts with the expression of these two genes. The *pyrB* gene encodes aspartate carbamoyltransferase, an enzyme that catalyzes the regulatory step of pyrimidine biosynthesis (Kafer & Thornburg, 1999). This gene was not induced by low temperature in wild-type cells, but was induced by low temperature in Δ *crhR* cells (Table 1). The expression of *gifA* and *gifB* genes for glutamine synthetase-inactivating factors 1 and 2, respectively, was slightly enhanced by low temperature in Δ*crhR*, whereas the same genes did not respond to low temperature in wild-type cells (Table 1). The *sll1611* gene for a hypothetical protein was induced in wild-type cells by low temperature, whereas the low temperature-induced expression of this gene was insignificant in Δ *crhR* cells (Table 1).

The expression of *groES, groEL1* and *groEL2* genes in wild-type cells was unaffected during incubation at 24 ^{o}C for the first 60 min. However, the expression of these genes was enhanced 3- to 4-fold during incubation at 24 ^{o}C from 60 min to 180 min (Table 1).

In contrast, the low temperature-inducible expression of the same genes was significantly repressed in Δ*crhR* cells during incubation at 24 ^{o}C for 60 min and it recovered to almost the original level at 180 min (Table 1). Functional complementation of CrhR *in trans* reversed the effect of Δ*crhR* mutation, although the recovery was incomplete (Table 1, the last column). To examine whether the incomplete recovery of low temperature-induced expression of genes was due to a low level of CrhR, we determined the level of CrhR by Western blotting (Fig. 4S). The result revealed that the level of CrhR was approximately the same between wild-type and *crhR*$^{+}$ cells.

Northern blotting analysis of the low temperature-inducible expression of the *groESL* and the *groEL2* genes

Changes in the low temperature-induced expression of *groESL* and *groEL2* genes due to the Δ*crhR* mutation were further examined by Northern blotting analysis (Fig. 3). We used primers for specific amplification of DNA fragment corresponding to the *groEL2* gene for detection of *groEL2* mRNA (Fig. 3a). As observed by the microarray analysis (Table 1), the expression of *groEL2* gene in wild-type cells was unaffected during incubation at 24 ^{o}C for 30 min. However, the expression of this gene was gradually enhanced during further incubation of wild-type cells at 24 ^{o}C, and approximately 5-fold increase in the levels of *groEL2* mRNA was observed during further incubation of wild-type cells at 24 ^{o}C for 180 min and this high level was maintained during incubation at 24 ^{o}C for 360 min in wild-type cells (Fig. 3a). In contrast, the low temperature-inducible expression of the *groEL2* gene was significantly repressed inΔ *crhR* cells during

366 incubation of cells at 24 ^{o}C for 30 min and 60 min, and was recovered to the original
367 level at 180 min (Fig. 3a).
368
369 The DNA probe that covered genes *groES* and *groEL1* of a dicistronic operon detected a
370 single band on Northern blotting (Fig. 3b). We observed a slight decrease in the mRNA
371 level of *groESL* during incubation at 24 ^{o}C for 30 min in wild-type cells. However, the
372 transcript level gradually increased during further incubation of wild-type cells at 24 ^{o}C.
373 We observed that the mRNA level increased approximately 4-fold during incubation of
374 wild-type cells at 24 ^{o}C for 180 min and maintained the high level at 360 min (Fig. 3b).
375 In contrast, the low temperature-inducible expression of *groESL* monocistronic operon
376 was significantly repressed inΔ *crhR* cells during incubation at 24 ^{o}C for 30 min and 60
377 min and recovered to the original and slightly higher than the original level in the Δ*crhR*
378 cells (Fig. 3b).
379
380 **CrhR was not involved in the heat-induced expression of the *groEL2* gene**
381
382 To examine whether CrhR is involved in the heat-induced expression of *groEL* genes, we
383 compared the heat-induced expression of the *groEL2* gene in wild-type and Δ*crhR* mutant
384 cells by Northern blotting analysis (Fig. 5S). The level of *groEL2* mRNA in wild-type
385 and Δ*crhR* cells increased 8 folds during incubation at 42 ^{o}C for 30 min and then, after a
386 slight decrerase, maintained a high level during further incubation at 42^{o}C for 240 min
387 (Fig. 5S). There was no significant difference in the time course of the mRNA levels
388 between wild-type and Δ*crhR* cells. These observations suggested that CrhR was not

involved in the regulation of heat-inducible expression of the *groEL2* gene in *Synechocystis.* Thus, it is very likely that CrhR is not a regulator of the gene expression of the *groEL2* gene at high temperatures.

Transcripts of *groESL* and *groEL2* were stabilized under low-temperature conditions

The level of mRNA is a result of balance between the rate of transcription and the rate of degradation. Therefore, the low temperature-induced changes in levels of *groESL* and *groEL2* mRNA, due to the mutation of the *crhR* gene, could be related to changes in the rate of transcription and/or changes in the stability of mRNAs. To elucidate whether CrhR regulates the *groESL* transcripts post-transcriptionally at the level of mRNA stability, we analyzed the decay kinetics of the *groEL2* transcript and *groESL* co-transcripts in the presence of rifampicin, an inhibitor of transcription. In this experiment, wild-type and Δ*crhR* mutant cells, which had been grown at 34 ^{o}C for 16 h (Figs. 4a and 5b), were incubated at 24^{o}C for 5 min (Figs. 4c and 4d) or for 180 min (Figs. 4e and 4f) before the assay of decay kinetics. In 34^{o}C-grown cells, mRNA of both *groESL* and *groEL2* degraded to zero at 30 min after addition of rifampicin; there was no difference between the rate of degradation of *groEL2* and *groESL* mRNAs between wild-type and Δ*crhR* cells (Figs. 4a and 4b). By contrast, the decay of *groEL2* and *groESL* transcripts was much slower in cells that had been incubated at 24^{o}C than that at 34^{o}C-grown cells; moreover, the degradation was faster in Δ*crhR* cells than in wild-type cells (Figs. 4c and 4d). The half-decay times for *groEL2* and *groESL* transcripts were 8 min and 12 min,

412 respectively, in wild-type cells, whereas they were 3.6 and 5 min, respectively, in Δ*crhR*
413 cells. These results indicated that the stability of *groEL2* and *groESL* transcripts
414 decreased to less than half of the original just after the shift in temperature from 34^{o}C to
415 24^{o}C (Figs. 4c and 4d). Taken together, the analysis of *groESL* expression by Northern
416 blotting and mRNA stability assays suggested that the marked decrease in levels of
417 mRNA during the initial phase of incubation at low temperature due to the Δ*crhR* mutant
418 cells (Figs. 3a and 3b) could be attributed to the decrease in stability of these transcripts
419 (Figs. 4c and 4d). These observations suggest that CrhR regulates the stability of *groEL2*
420 and *groESL* mRNAs at the early phase of incubation at low temperature.
421
422 Figs. 4e and 4f depict the stability of *groEL2* transcripts and *groESL* co-transcripts in
423 wild-type and Δ*crhR* cells after incubation of these cells at 24^{o}C for 180 min. The
424 *groEL2* and *groESL* transcripts were equally stable (Figs. 4e and 4f). But, at this time
425 point, we observed a significant increase in levels of *groEL2* and *groESL* transcripts in
426 wild-type cells (Figs. 3a and 3b). In contrast, in Δ*crhR* mutant cells, there was no
427 increase in levels of *groEL2* transcript and a slightly higher than original level of *groESL*
428 transcript was observed (Figs. 3a and 3b). These findings suggested that the differential
429 expression of *groEL2* and *groESL* genes during the late phase of incubation at low
430 temperature (Figs. 3a and 3b) wa not due to the differential stability of transcripts of these
431 genes (Figs. 4e and 4f). It seems likely that CrhR might also be involved in the
432 regulation of *groEL2* and *groESL* expression at the level of transcription during the late
433 phase.
434

Low temperature-inducible synthesis of GroEL depends on the presence of CrhR

To examine whether the level of GroEL proteins reflects the alteration in the expression of *groESL* genes at the transcript level, we followed, by Western blotting analysis, changes in the level of GroEL1 and GroEL2 in wild-type and Δ*crhR* cells during incubation at low temperature. Since antibodies that had been raised against GroEL1 of *E. coli* immunologically reacted with both GroEL1 and GroEL2 of *Synechocystis*, our results demonstrated changes in the level of GroEL1 plus GroEL2 (hereafter, GroEL). As shown in Fig. 5, the level of GroEL in wild-type cells increased 1.5-fold during incubation at 24^{o}C for the first 60 min and about 2 fold at 180 min. In contrast, the level of GroEL reduced to 50% in Δ*crhR* mutant cells during incubation at 24^{o}C for 60 min and then recovered approximately to the original level during further incubation of cells at 24^{o}C for 300 min. As a result, the mutation of *crhR* abolished the low temperature-inducible accumulation of GroEL (Fig. 5). The phenotype of slow growth at low temperature of Δ*crhR* cells could be due to the lowered level of GroEL proteins at low temperature.

In conclusion, our results suggest that, in *Synechocystis* cells, RNA helicase CrhR regulates the low-temperature inducibility of molecular chaperonins, GroES, GroEL1 and GroEL2, which are, in turn, essential for growth at low temperatures. Low-temperature inducibility of genes for the heat-shock proteins has been reported in other organisms. In an Antarctic bacterium *Oleispira antarctica*, the expression of cold-inducible expression of GroES and GroEL is important in acclimatization to low temperatures (Ferrer *et al.*,

2003). In the thermophilic cyanobacterium *Thermosynechococcus elongatus*, GroEL2 is essential for the acclimatization to the low temperatures, as well as to high temperatures (Sato *et al.*, 2008).

There may be two possible mechanisms for the functional role of chaperonins in acclimatization to low temperature. First, low temperature may cause denaturation and/or aggregation of some specific proteins in the cell (Ray, 2006). Proper folding and maintenance of structure are essential for the enzymatic activity of proteins at low temperatures (Strocchi *et al.*, 2006). Since chaperonins assist the folding of proteins, their expression at low temperatures might protect the proteins against denaturation and aggregation.

The second proposed mechanism at low temperatures involving GroES and GroEL may be the quality control of proteins. GroESLs enhance degradation of truncated proteins that are produced due to inhibition of translation at low temperatures (Kandror *et al.*, 1994). The low temperature-inducible trigger factor (TF) binds to GroEL and enhances GroEL's affinity to unfolded proteins, and thus promotes degradation of certain polypeptides (Kandror & Goldberg, 1997). Thus, the folding and degradation of proteins seem to play a major role in the process of acclimatization to low temperature. Chaperonins, in association with other proteins, contribute to such quality control of proteins. Evidences support the possibility of chaperonin involvement in both, stimulation of protein refolding and enhancement of protein degradation at low temperatures.

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Table 1. Genes whose inducibility by low temperature was affected by the mutation in Δ*crhR*

Wild-type and Δ*crhR* cells were grown at 34 °C for 16 h and then incubated at 24 °C for 20, 60 and 180 min. Each value indicates the ratio of levels of the mRNA from cold-stressed cells to those from control cells. The values shown are the results of two independent experiments with the range of experimental deviations. The numbering of ORFs corresponds to that of Kaneko et al. (1996).

The complete list of gene expression in wild-type and Δ*crhR* mutant can be accessed at http://www.genome.ad.jp/kegg-bin/get_htext?htext=Exp_DB&hier=1

ORF no.	Gene	Product	24 °C / 34 °C						
			Wild-type cells			Δ*crhR* cells			*crhR*$^+$ cells
			20 min	60 min	180 min	20 min	60 min	180min	180 min
Genes whose inducibility by low temperature was depressed by mutation of the *crhR* gene									
sll1611		Hypothetical protein	4.0±0.4	8.4 ±1.5	6.7 ±1.3	2.1±0.1	2.3 ±0.1	1.9±0.1	3.7±0.02
slr2076	*groEL1*	60 kDa chaperonin 1	0.6±0.0	0.7±0.0	4.4 ±1.2	0.2±0.0	0.2±0.0	1.8±0.2	3.5±0.12
sll0416	*groEL2*	60 kDa chaperonin 2	0.6±0.1	0.7±0.1	3.1±0.4	0.2±0.0	0.2±0.0	1.1±0.1	2.7±0.07
slr2075	*groES*	10 kDa chaperonin	0.5±0.1	0.5±0.0	3.9±1.0	0.2±0.0	0.2±0.0	1.6±0.5	2.9±0.12
Genes in whose inducibility by low temperature was enhanced by mutation of the *crhR* gene									
slr0082		Hypothetical protein	9.3±0.8	2.3±0.1	1.0±0.1	11.6±0.8	8.7±1.1	8.8±0.2	1.3±0.04
slr1476	*pyrB*	Aspartate carbomoyl transferase	1.9±0.2	1.5±0.1	1.4±0.4	4.1±0.9	3.8±0.9	3.7±1.1	1.2±0.16
ssl1911	*gifA*	Glutamine synthetase inactivating factor	1.3±0.7	0.9±0.3	0.4±0.2	2.4±0.3	3.1±0.6	2.3±0.7	1.5±0.01
sll1515	*gifB*	Glutamine synthetase inactivation factor	1.5±0.5	1.5±0.1	0.9±0.4	3.4±0.9	2.7±0.4	1.8±0.5	2.2±0.08

Legends to Figures

Fig. 1. Northern-blotting and Western-blotting analyses of changes in the expression of the *crhR* gene upon the downward shift in temperature in wild-type cells of *Synechocystis*. (a) Northern blotting of *crhR* mRNA. Total RNA was extracted from wild-type cells that had been grown at 34 ^{o}C for 16 h or had been grown at 34 ^{o}C for 16 h and then incubated at 24 ^{o}C for 10, 20, 30, 40, 50, 60 and 120 min. Aliquots (20 μg) of the extracted RNA were electrophoresed on 1.2% agarose gels that contained 1.4 M formaldehyde. (b) Western blotting of CrhR. Soluble proteins was extracted from wild-type cells that had been grown at 34 ^{o}C for 16 h or had been grown at 34 ^{o}C for 16 h and then incubated at 24 ^{o}C for 30, 60, 180 and 300 min. Samples equivalent to 25 μg proteins was loaded in each well of polyacrylamide gel (12%) that contained 0.1% SDS. CrhR antibody produced in rat was used to detect CrhR on the blot. (c) Quantitative expression of *crhR* mRNA and CrhR upon the downward shift in temperature.

Fig. 2. Mutation and complementation of the *crhR* gene affected growth profiles at low temperature. Wild-type cells (●), Δ*crhR* mutant cells (○) and the complemented *crhR*$^{+}$ cells (Δ) were grown photoautotrophically at 70 μmol photons $m^{-2}s^{-1}$ at 34 ^{o}C (a) or 24 ^{o}C (b). Cell growth was monitored by measuring the apparent absorbance at 730 nm. Similar results were obtained in three independent experiments, and the data represented as mean ±SD.

622 **Fig. 3.** Northern blotting analysis of the low temperature-induced expression of *groESL*
623 and *groEL2* genes in wild-type and Δ*crhR* cells. RNAs were extracted from wild-type
624 and Δ*crhR* cells that had been grown at 34 °C for 16 h and then incubated at 24 °C for
625 designated periods of time. Aliquots (15 μg) of the extracted RNA were fractionated on
626 1.2% agarose gels that contained 1.4 M formaldehyde. (a) The *groEL2* gene. A 1646 bp
627 DNA fragment that included the *groEL2* gene was amplified using primer set 5'TTT
628 CCT TTA AGG ATG AAT CCA G3' and a reverse primer 5'TTA CATCAT GCC CAT
629 GCC3' by PCR and used as a probe. (b) The *groESL* operon. A 954-bp DNA fragment
630 that included both *groES* and *groEL* open reading frames was amplified by PCR and used
631 as a probe. ○, Wild-type cells; ●, Δ*crhR* cells. For normalization, extents of signals
632 obtained with *groEL2* or *groESL* were compared with signals of the *rnpB* mRNA.
633 Similar results were obtained in two independent experiments, and the figure shows the
634 results of one of these experiments.
635
636 **Fig. 4.** Effects of *crhR* mutation on the stability of *groEL2* transcript and the *groESL* co-
637 transcript. (a) and (b) Levels of *groEL2* and *groESL* transcripts in wild-type and Δ*crhR*
638 cells, respectively, after cells had been grown at 34 °C for 16 h. (c) and (d) Levels of
639 *groEL2* and *groESL* transcripts in wild-type and Δ*crhR* cells, immediately after the shift
640 in temperature from 34 °C to 24 °C. (e) and (f) Levels of *groEL2* and *groESL* transcripts
641 in wild-type and Δ*crhR* cells, after the cells had been grown at 34 °C for 16 h and then
642 incubated at 24 °C for 180 min. ○, Wild-type cells; ●, Δ*crhR* cells. Rifampicin (100 μg
643 ml^{-1}) was added to the control or low temperature-treated cells and then the cells were
644 harvested at various periods of time for extraction of RNAs. Signals detected with a

luminescence image analyzer (LAS-1000; Fuji-Photo Film) were used for calculating the relative abundance of *groEL2* and *groESL* transcripts and were plotted on a logarithmic scale against the time after addition of rifampicin.

Fig. 5. Changes in the level of GroEL in wild-type and Δ*crhR* cells after a downward shift in temperature. Soluble proteins were extracted from wild-type and Δ*crhR* cells that had been grown at 34 ^{o}C and then incubated at 24^{o}C for 30, 60, 180, and 300 min. Samples equivalent to 25 μg of proteins were loaded in each well. Experiment was performed twice and the results are presented as a mean of the two independent experiments.