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

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The \textit{crhR} gene for RNA helicase, CrhR, was one of the most highly induced genes when the cyanobacterium \textit{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 \textit{crhR} gene by replacement with a spectinomycin-resistance gene cassette. The resultant \textit{ΔcrhR} mutant exhibited a phenotype of slow growth at low temperatures. DNA microarray analysis of the genome-wide expression of genes, and Northern and Western blotting analyses indicated that mutation of the \textit{crhR} gene repressed the low-temperature-inducible expression of heat-shock genes \textit{groEL1} and \textit{groEL2}, at the transcript and protein levels. The kinetics of the \textit{groESL} co-transcript and the \textit{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 \textit{Synechocystis} cells to low temperatures.

INTRODUCTION

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

We have been working on the mechanisms of acclimatization to low-temperature stress of the cyanobacterium \textit{Synechocystis} sp. PCC 6803 (hereafter, \textit{Synechocystis}). Studies using DNA microarrays demonstrated that low-temperature stress induced the expression of a large number of genes, including 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 \textit{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 believed to optimize the fluidity in membranes at low temperatures (Los \textit{et al.}, 2008).

ChrR in vitro is active in unwinding, annealing and exchanging RNA strands, its function during acclimatization of Synechocystis cells to low temperature has not been demonstrated.

RNA helicases are ubiquitously distributed in all the biological kingdoms (Rocak & Linder, 2004). Extensive studies have demonstrated that they are active in modulating the secondary structure of RNAs by unwinding RNAs in an ATP-dependent manner (Tanner & Linder, 2001). They participate in various cellular processes in which RNAs are involved. In model experimental organisms, such as E. coli, a cold-inducible RNA helicase, CsdA, has been suggested to participate in the assembly of ribosomes (Peil et al., 2008). Another RNA helicase is a component of the ‘cold-shock degradosome complex’ and enhances the degradation of mrnas by unwinding the secondary structures so as to facilitate cleavage of mRNA by RNase E (Carpousis et al., 1999; Prud’honhomme-Genereux et al., 2004).

The cyanobacterium Anabaena sp. PCC 7120 contains two genes for RNA helicases; the crhC gene is induced only under low-temperature stress whereas the crhB gene is induced by salt, low-temperature and light stress, as well as by nitrogen limitation (Chamot et al., 1999). Biochemical characterization and cellular localization of CrhC suggested that it is a membrane-bound protein and may be involved in translocation of proteins across the plasma membrane under low-temperature conditions (El-Fahmawi & Owtram, 2003).

In this work, we aimed to elucidate the functional role of RNA helicase, ChrR, 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 groEL2 and groEL2, at 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 °C in BG-11 medium (Stanier et al., 1971), buffered with 20 mM HEPES/NaOH (pH 7.5), under continuous illumination from incandescent lamps, as described previously (Wada & Murata, 1989). ΔcrhR mutant cells (see below), in which the crhR gene had been replaced by the spectinomycin resistance gene (Sp') cassette in low-temperature stress whereas the crhR mutant cells (see below), in which the crhR gene had been replaced by the spectinomycin resistance gene (Sp') 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. ΔcrhR mutant cells were then transferred to the above-mentioned BG-11 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 ΔcrhR mutant cells. We generated a ΔcrhR mutant by replacing the crhR gene (slr0083) by the Sp' cassette (see Supplementary Fig. S1, available with the online version of this paper); 767 bp upstream and 960 bp downstream flanking regions of ORF slr0083 from the genomic DNA were amplified using primer sets UF (5'-AAT CTA GAG TCG ATA TTC CTT GGA TTC GTA TT-3')/UR (5'-AAA GGC CTG AGC GTT TAG TGG GCA AAT AAT T-3') and DF (5'-AAA GGC CT AAC TCC TCC AGA ACT AAG ACC-3')/DR (5'-AAG AGC TCC ATG GAA CCC ATT GAC GTA GAG-3'), respectively. An XbaI site and a SacI site (underlined) were created in primers UF and DR, respectively, during primer synthesis. A StuI site (underlined) was created in the primers UR and DF. The PCR fragments generated using UF-UR and DF-DR primer sets were cloned separately into pT7Blue F-A cloning vector and named pTcrh767 and pTcrh960, respectively. The DF-DR fragment released from pTcrh960 after digestion with StuI and SacI restriction enzymes was ligated to the same sites on pTcrh767. The resultant construct was named pTcrh-. The Dral-digested Omega Sp' cassette was cloned onto the StuI site of pTcrh- construct by blunt end ligation. The final construct, in which the slr0083 ORF had been replaced by the Omega Sp' 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 (Supplementary Fig. S1b). This analysis indicated that the wild-type copy of the crhR gene had been completely replaced by the mutated copy in ΔcrhR cells. The resultant mutant was designated ΔcrhR.

Complementation of ΔcrhR mutation. We complemented ΔcrhR cells with a derivative of the cyanobacterial autonomously replicating plasmid pVZ321 (Zinchenko et al., 1999) harbouring the crhR gene. A DNA fragment that included the crhR gene and its upstream 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 TCC TCG AGG AGT TAT TTT TGG CGT ACG CT-3'. A HindIII and an Xhol site (underlined) were created in the forward and reverse primers, respectively. The product amplified by PCR was digested with HindIII and Xhol and the resulting fragment of 2007 bp was inserted into pVZ321 which had been digested with the same enzymes. The resultant plasmid was introduced into ΔcrhR cells by triparental gene transfer (Zinchenko et al., 1999). The complemented strain was designated 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 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) to remove contaminating DNA. cDNAs, labelled with fluorescent dyes (Cy3 and Cy5; Amersham Pharmacia Biotech), were prepared from 10 µg total RNA with an RNA Fluorescence Labelling Core kit (M-MLV, version 2.0; Takara) 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), which covered 3079 of the 3168 ORFs (97% of total genes except transposon-related genes) of the Synechocystis genome. Hybridization of the labelled cDNA to the 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
喷雾至分析扫描仪（GMS418；Affimetrix）。每个信号被量化与ImaGene版本4.0程序（BioDiscovery）。该信号从每种基因在微阵列上被正交化，与所有基因的信号强度进行比较，以确定信号强度的最大值与信号强度的平均值的比例。这样就可以计算出每个基因表达水平的相对值。Northern blotting analysis. Total RNA was extracted from cells, and Northern blotting analysis was performed as described by Los et al. (1997). In the case of mRNA stability experiments, wild-type and ΔcrhR 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 the 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) 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 analyser (LAS-1000; Fuji-Photo Film).

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′-GCGTCGAC-GACTAATACT-TTACTGTTGGCGATCACTATAG-3′ and the reverse primer 5′-GGTTGCAC-TTACTGTGCGATCATGACTAG-3′ and purified by agarose gel electrophoresis. The amplified ORF of crhR was eluted from the gel and was inserted into pET-28a (+) at the NdeI and SalI sites to generate pET-CrhR. The N-terminally His-tagged CrhR protein was expressed in BL21(DE3)pLysS, which had been transformed with pET-CrhR, and was purified using HIS-Select Nickel Affinity gel (Sigma – P6611) according to the supplier’s instruction. The expression of CrhR protein was induced by addition of 400 μM (final concentration) IPTG. Bacterial cells were collected by centrifugation at 10 000 g for 10 min and pelleted cells were disrupted with a sonic oscillator (model, UV2070; probe, MS-72; Bandelin Electronic) operated for 10 min at 50 % power, with 1 min pulse interval, in 100 mM Tris/HCl (pH 8.0) and 200 mM NaCl. Insoluble materials (precipitates) were removed by centrifugation at 10 000 g for 20 min at 4 °C. The supernatant was loaded onto a HIS-Select Nickel Affinity column. 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 containing 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).

Western blotting analysis. Soluble proteins were extracted from Synechocystis cells by mechanical disruption of cells using glass beads (106 μm diameter; Sigma). Synechocystis cells in 200 μl 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 ensure maximum disruption of cells. Then, mechanically disrupted cells were centrifuged at 25 000 g for 20 min at 4 °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 gels on an equal protein basis (25 μg soluble protein in each well). After electrophoresis, the separated proteins were blotted onto a PVDF membrane (Millipore) in a semi-dry transfer apparatus (Atto). 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). 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 (catalogue no. G-6532) as the primary antibody and horseradish peroxidase antibody raised in donkey against rabbit immunoglobulin G as the secondary antibody. A luminescence image analyser (LAS-1000; Fuji-Photo Film) was used to monitor signals from blotted membranes.

RESULTS AND DISCUSSION

Expression of the crhR gene is 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. 1(a) shows changes in the level of crhR mRNA during exposure of Synechocystis cells, which had been grown at 34 °C, to 24 °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 transiently increased the level of crhR transcript, with the maximum attained at 30 min during the incubation at 24 °C (Fig. 1a, c).

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 °C of Synechocystis cells which had been grown at 34 °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 °C from growth conditions. Our previous DNA microarray study demonstrated that almost all 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 two-component signal transduction.
Mutation of the crhR gene and complementation of the ΔcrhR mutant

In order to confirm that the changes in phenotype seen in the ΔcrhR mutant, as will be discussed in the next sections, were caused by mutation of the crhR gene, we compared the growth profile of wild-type, ΔcrhR mutant and crhR-complemented crhR+ cells at 24 °C and 34 °C. At 34 °C these three types of cell revealed similar profiles of growth (Fig. 2a). At 24 °C, however, growth of ΔcrhR 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 ΔcrhR cells in trans with a functional CrhR expressed from pVZ-pcrhR (crhR+ strain) restored growth at the low temperature, which was similar to that of wild-type cells (Fig. 2b). The functional complementation of ΔcrhR cells by the crhR gene clearly demonstrated that CrhR plays an important role in the physiology of Synechocystis cells at low temperatures.

Fig. 1. Northern and Western blotting analyses of changes in the expression of the crhR gene upon 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 °C for 16 h or had been grown at 34 °C for 16 h and then incubated at 24 °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 were extracted from wild-type cells that had been grown at 34 °C for 16 h or had been grown at 34 °C for 16 h and then incubated at 24 °C for 30, 60, 180 and 300 min. Samples equivalent to 25 μg proteins were loaded in each well of a 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 (means ± SD of three independent experiments).

Fig. 2. Mutation and complementation of the crhR gene affected growth profiles at low temperature. The wild-type (●), the ΔcrhR mutant cells (○) and the complemented crhR+ strain (△) were grown photoautotrophically (70 μmol photons m⁻² s⁻¹) at 34 °C (a) or 24 °C (b). Growth was monitored by measuring the OD₇₅₀. Similar results were obtained in three independent experiments, and the data are represented as means ± SD.
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 ressembles translation by unwinding the base-paired regions of mRNA that 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 analysed the genome-wide expression of genes in the ΔcrhR mutant cells by a 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 (growth temperature, 34°C; light intensity, 70 μmol photons m⁻² s⁻¹; CO₂ 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 induction factor 2.0 or below 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 ΔcrhR cells had no significant effect on the genome-wide expression of genes under isothermal conditions at 34°C (Supplementary Fig. S2).

Next, we performed DNA microarray analysis to investigate whether the mutation in ΔcrhR cells would have any effect on the low-temperature-induced expression of genes. Supplementary Fig. S3(a), (b) and (c) represent the gene expression profiles in wild-type cells after exposure of cells to low temperature for 20, 60 and 180 min, respectively. Supplementary Fig. S3(d), (e) and (f) represent the gene expression profiles in ΔcrhR 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 ΔcrhR. The genes for molecular chaperonins, groES, groEL and groEL2, are indicated by black data points in the scatter plots in Supplementary Fig. S3.

Table 1 lists genes whose low-temperature inducibility was significantly affected by the 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 from 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 is a regulatory mechanism that interacts with the expression of these two genes. The *pyrB* gene...

### Table 1. Genes whose inducibility by low temperature was affected by the mutation in ΔcrhR

<table>
<thead>
<tr>
<th>ORF no.</th>
<th>Gene</th>
<th>Product</th>
<th>24°C/34°C</th>
<th>24°C/34°C</th>
<th>24°C/34°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wild-type cells</td>
<td>ΔcrhR cells</td>
<td>crhR⁺ cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 min</td>
<td>60 min</td>
<td>180 min</td>
</tr>
<tr>
<td>sll1611</td>
<td>Hypothetical protein</td>
<td>4.0 ± 0.4</td>
<td>8.4 ± 1.5</td>
<td>6.7 ± 1.3</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>slr2076</td>
<td>groEL1</td>
<td>60 kDa chaperonin 1</td>
<td>0.6 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>4.4 ± 1.2</td>
</tr>
<tr>
<td>sll0416</td>
<td>groEL2</td>
<td>60 kDa chaperonin 2</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>slr2075</td>
<td>groES</td>
<td>10 kDa chaperonin</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.0</td>
<td>3.9 ± 1.0</td>
</tr>
<tr>
<td>slr0082</td>
<td>Hypothetical protein</td>
<td>9.3 ± 0.8</td>
<td>2.3 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>11.6 ± 0.8</td>
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<tr>
<td>slr1476</td>
<td>Aspartate carbamoyltransferase</td>
<td>1.9 ± 0.2</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.4</td>
<td>4.1 ± 0.9</td>
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<tr>
<td>ssl1911</td>
<td>gifA</td>
<td>Glutamine synthetase inactivating factor</td>
<td>1.1 ± 0.7</td>
<td>0.9 ± 0.3</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>sll1515</td>
<td>gifB</td>
<td>Glutamine synthetase inactivating factor</td>
<td>1.5 ± 0.5</td>
<td>1.5 ± 0.1</td>
<td>0.9 ± 0.4</td>
</tr>
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</table>
gene encodes aspartate carbamoyltransferase, an enzyme that catalyses 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 ΔcrhR 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 ΔcrhR, 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 ΔcrhR cells (Table 1).

The expression of the groES, groEL1 and groEL2 genes in wild-type cells was unaffected during incubation at 24 °C for the first 60 min. However, the expression of these genes was enhanced three- to fourfold during incubation at 24 °C from 60 min to 180 min (Table 1). In contrast, the low-temperature-inducible expression of the same genes was significantly repressed in ΔcrhR cells during incubation at 24 °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 ΔcrhR mutation, although the recovery was incomplete (Table 1, 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 (Supplementary Fig. S4). The result revealed that the level of CrhR was approximately the same for wild-type and crhR+ cells.

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

Changes in the low-temperature-inducible expression of groESL and groEL2 genes due to the ΔcrhR mutation were further examined by Northern blotting analysis (Fig. 3). We used primers for specific amplification of a 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 the groEL2 gene in wild-type cells was unaffected during incubation at 24 °C for 30 min. However, the expression of this gene was
gradually enhanced during further incubation of wild-type cells at 24 °C, and an approximately fivefold increase in the levels of groEL2 mRNA was observed during further incubation of wild-type cells at 24 °C for 180 min and this high level was maintained during incubation at 24 °C for 360 min in wild-type cells (Fig. 3a). In contrast, the low-temperature-inducible expression of the groEL2 gene was significantly repressed in ΔcrhR cells during incubation of cells at 24 °C for 30 min and 60 min, and had recovered to the original level at 180 min (Fig. 3a).

The DNA probe that covered genes groES and groEL1 of a dicistronic operon detected a single band on Northern blotting (Fig. 3b). We observed a slight decrease in the mRNA level of groESL during incubation at 24 °C for 30 min in wild-type cells. However, the transcript level gradually increased during further incubation of wild-type cells at 24 °C. We observed that the mRNA level increased approximately fourfold during incubation of wild-type cells at 24 °C for 180 min and maintained the high level at 360 min (Fig. 3b). In contrast, the low-temperature-inducible expression of the groESL monocistronic operon was significantly repressed in ΔcrhR cells during incubation at 24 °C for 30 min and 60 min and then recovered to the original and slightly higher than the original level in the ΔcrhR cells (Fig. 3b).

**CrhR is not involved in the heat-induced expression of the groEL2 gene**

To examine whether CrhR is involved in the heat-induced expression of groEL genes, we compared the heat-induced expression of the groEL2 gene in wild-type and ΔcrhR mutant cells by Northern blotting analysis (Supplementary Fig. S5). The level of groEL2 mRNA in wild-type and ΔcrhR cells increased eightfold during incubation at 42 °C for 30 min and then, after a slight decrease, maintained a high level during further incubation at 42 °C for 240 min (Supplementary Fig. S5). There was no significant difference in the time-course of the mRNA levels between wild-type and ΔcrhR 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 expression of the groEL2 gene at high temperatures.

**Transcripts of groESL and groEL2 are 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 analysed 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 ΔcrhR mutant cells, which had been grown at 34 °C for 16 h (Fig. 4a, b), were incubated at 24 °C for 5 min (Fig. 4c, d) or for 180 min (Fig. 4e, f) before the assay of decay kinetics. In cells grown at 34 °C, mRNA of both groESL and groEL2 degraded to zero at 30 min after the addition of rifampicin; there was no difference between the rate of degradation of groEL2 and groESL mRNAs between wild-type and ΔcrhR cells (Fig. 4a, b). By contrast, the decay of groEL2 and groESL transcripts was much slower in cells that had been incubated at 24 °C than those grown at 34 °C; moreover, the degradation was faster in ΔcrhR cells than in wild-type cells (Fig. 4c, d). The half-decay times for groEL2 and groESL transcripts were 8 min and 12 min, respectively, in wild-type cells, whereas they were 3.6 and 5 min, respectively, in ΔcrhR cells. These results indicated that the stability of groEL2 and groESL transcripts decreased to less than half of the original just after the shift in temperature from 34 °C to 24 °C (Fig. 4c, d). Taken together, the analysis of groESL expression by Northern blotting and mRNA stability assays suggested that the marked decrease in levels of mRNA during the initial phase of incubation at low temperature in the ΔcrhR mutant cells (Fig. 3a, b) could be attributed to the decrease in stability of these transcripts (Fig. 4c, d). These observations suggest that CrhR regulates the stability of groEL2 and groESL mRNAs at the early phase of incubation at low temperature.

Fig. 4(e) and (f) depict the stability of groEL2 transcripts and groESL co-transcripts in wild-type and ΔcrhR cells after incubation of these cells at 24 °C for 180 min. The groEL2 and groESL transcripts were equally stable (Fig. 4e, f). But, at this time point, we observed a significant increase in levels of groEL2 and groESL transcripts in wild-type cells (Fig. 3a, b). In contrast, in ΔcrhR mutant cells, there was no increase in levels of groEL2 transcript and a slightly higher than original level of groESL transcript was observed (Fig. 3a, b). These findings suggested that the differential expression of groEL2 and groESL genes during the late phase of incubation at low temperature (Fig. 3a, b) was not due to the differential stability of transcripts of these genes (Fig. 4e, f). It seems likely that CrhR might also be involved in the regulation of groEL2 and groESL expression at the level of transcription during the late phase.

**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 ΔcrhR 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°C for the first 60 min and about twofold at 180 min. In contrast, the level of GroEL reduced to 50% in ΔcrhR mutant cells during incubation at 24°C for 60 min and then recovered to approximately the original level during further incubation at 24°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 ΔcrhR cells could be due to the lowered level of GroEL proteins at low temperature.

**Concluding remarks**

Our results suggest that, in *Synechocystis* cells, RNA helicase CrhR regulates the low-temperature inducibility of molecular chaperons 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 the Antarctic bacterium *Oleispira antarctica*, the 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 acclimatization to low temperatures, as well as to high temperatures (Sato et al., 2008).

There are two possible mechanisms for the functional role of chaperons 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 enzymic activity of proteins at low temperatures (Strocchi et al., 2006). Since chaperons assist the folding of proteins, their expression at low temperatures might protect the proteins against denaturation and aggregation.

The second possible mechanism at low temperatures involving GroES and GroEL is 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 hence 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. Evidence supports the possibility of chaperonin involvement in both stimulation of protein refolding and enhancement of protein degradation at low temperatures.

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