Expression and function of a *groEL* paralog in the thermophilic cyanobacterium *Thermosynechococcus elongatus* under heat and cold stress

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Abstract

Cyanobacterial genomes generally contain two *groEL* genes, referred to as *groEL1* and *groEL2*. The purpose of this study is to elucidate a role of *groEL2* in the adaptation of the thermophilic cyanobacterium *Thermosynechococcus elongatus* to hot environments. Both *groEL* genes were found to be heat-induced, while only *groEL2* was greatly cold-induced. Primer extension and gel mobility shift analyses indicated that transcriptional regulation of *groEL2* is different from that of *groESL1*. The *groEL2* gene was dispensable under normal growth conditions at 50˚C as a *groEL2* disruptant was viable. This *groEL2* mutant was highly sensitive to both high and low temperatures.

Key words: CIRCE, cold stress, Cpn60, GroEL, heat-shock protein, HrcA, thermophilic cyanobacterium

Footnotes

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1. Introduction

The genomes of many α-proteobacteria, cyanobacteria, and actinobacteria contain multiple copies of *groEL* [1, 2, 3, 4]. The presence of at least one *groEL* gene is essential [5, 6, 7, 8] because it provides the usual housekeeping chaperone functions exemplified by *E. coli* GroEL [9]. It is not clear what roles are fulfilled by the other *groEL* genes. Recently, it has been established that a non-essential *groEL1* gene plays a specific role in mycolic acid biosynthesis during biofilm formation in Mycobacteria [10]. *In vitro* experiments have shown that the three GroEL homologues from *Rhizobium leguminosarum* have distinct properties, differing, for instance, in stability and refolding activities [11].

Cyanobacteria are photosynthetic prokaryotes with highly differentiated membranes called thylakoid membranes. They successfully compete with other organisms in many environments such as subzero lakes in Antarctica and hot springs. Two distinct *groEL* genes, designated as *groEL1* and *groEL2*, generally exist in a cyanobacterial genome (the CyanoBase database, http://bacteria.kazusa.or.jp/cyanobase/). Interestingly, there is only one *groES* per genome, and one of the two *groEL* genes, *groEL2*, usually does not form an operon with the *groES* gene, although there are exceptions, such as the *groEL*s from *Gloeobacter violaceus* PCC 7421, which is, according to phylogenetic analysis, a member of an early branching lineage (CyanoBase). This strain lacks thylakoid membranes. The *Gloeobacter* genome contains two *groEL* genes both of which have *groES* immediately upstream of each *groEL*. It appears that one of the two *groESL* operons has lost its *groES* during the evolutionary cyanobacterial diversification.

The two *groEL* genes in cyanobacteria such as *Synechococcus vulcanus* and *Synechocystis* sp. strain PCC 6803 are expressed to different degrees [12,13]. *In silico* analysis [14] indicates that negative regulation by the CIRCE operator/HrcA repressor

system [15] is generally involved in the regulation of *groESL1* operon expression, while it is not involved in the control of *groEL2* expression in some cyanobacteria. This is because CIRCE is not present upstream of some *groEL2* genes, including the one in the thermophilic cyanobacterium *Thermosynechococcus elongatus*, the organism used for this study, while it is highly conserved among the *groESL1* operons [14]. We showed that HrcA regulates the CIRCE-containing *groE* genes of the cyanobacterium *Synechocystis* sp. PCC 6803 negatively [16]. In addition to the negative regulation, *Synechocystis groE* expression is regulated by a positive, light-responsive mechanism mediated by novel DNA elements designated as K- and N-boxes [14].

Although it is unclear whether the *groEL* paralogs play specialized roles in cyanobacterial cells, results of complementation tests in *E. coli* suggest that the two paralogs are functionally different. The *E. coli groEL* mutant can almost be returned to wild type by complementation with the cyanobacterial *groEL1* gene, but not with the *groEL2* gene [12, 13, 17]. Thus, the cyanobacterial *groESL1* operon may also play an essential housekeeping role, while the function of *groEL2* is enigmatic. It is impossible to disrupt *groESL1* of *Synechococcus elongatus* strain PCC 7942 [18], indicating that the operon is indispensable for the cyanobacterial growth. We hypothesized that *groEL2* in cyanobacteria evolved to play a non-essential role under normal growth conditions, but become essential under stress conditions. For the present study, we tested this hypothesis, using a themophile, *T. elongatus*. This cyanobacterium requires high temperatures for growth. It is not known how it has adapted to high temperature during evolution. In order to test the above hypothesis, the level of expression of *T. elongatus groEL2* was analyzed at high and low temperatures, and the importance of the *groEL2* expression was evaluated by characterizing a newly constructed *groEL2* mutant. The GroEL2 diversification may play an important role in expanding the cyanobacterial habitat range

into a hot environment.

2. Materials and methods

Organisms and culture conditions. Unless otherwise indicated, *T. elongatus* BP-1 cells were grown at 50˚C under a light intensity of 50 μE/m^2/s in liquid BG-11 medium or on BG-11 plates containing 1.5% (w/v) agar and 0.3% (w/v) sodium thiosulfate [14]. The liquid culture was continuously illuminated and bubbled with air containing 5% CO_2 until sampling.

Northern blot analysis. Total RNA was extracted from cells as previously described [14]. Suitable fragments for the preparation of the *groES*- (to detect transcripts of the *groESL1* bicistronic operon), *groEL2*-, and *rnpB*-specific probes for detection of the corresponding RNA from *T. elongatus* were amplified by polymerase chain reaction (PCR) with genomic DNA as the template and appropriate pairs of primers as listed in Table 1. For the amplification of the DNA fragments, an antisense oligonucleotide primer containing T7 RNA polymerase promoter was used (Table 1). This was directly used for the synthesis of the digoxigenin-labeled complementary RNA probe. For the preparation of the *hspA*-specific RNA probe, a 0.9-kbp DNA fragment containing the *S.vulcanus hspA* gene was amplified by PCR with pBS-HSPAX [19] as a template and a pair of primers, Tel_hspA_06 and Tel_hspA_07 (Table 1). The DNA fragment was cloned into pBluescript II (Stratagene), resulting in pBS-HSPA0706. After digesting pBS-HSPA0706 with *Hin*cII, T7 RNA polymerase promoter was used for the synthesis of the digoxigenin-labeled complementary RNA probe. Northern blot analyses with the probes were performed as described previously [14].

Primer extension analysis. The 5'-ends of synthetic oligonucleotides (Table 1), which were complementary to a region downstream of the translational initiation codon of

groESL1 (Tel_groES_PriEx_1) and regions upstream and downstream of the translational initiation codon of *groEL2* (Tel_cpn60_PriEx_1, Tel_cpn60_PriEx_2, and Tel_cpn60_PriEx_3) were labeled with [γ-^{32}P] ATP and used as primers. Primer extension reactions were performed as described previously [20].

Construction of mutants. A region of the *T. elongatus* genome containing *groEL2* was amplified by PCR with the primers Tel_cpn60_02_T7 and Tel_cpn60_-885_F (Table 1). The PCR product was cloned into pT7Blue T-vector (Novagen). The resulting plasmid DNA, pTel_cpn60, contained a unique *Hpa*I restriction site in the coding region of the *groEL2* gene. A chloramphenicol-resistant gene cassette was inserted into the restriction site in the forward direction (the same gene orientation as that of *groEL2*). The cassette was isolated by digesting pTCm56 [21] with *Hin*cII. The construct, pTel_cpn60::Cm, was electroporated into cells of *T. elongatus* to obtain transformed cells having a correctly targeted homologous recombination, and these were segregated for a few generations by single colony selection on BG-11 agar plates to isolate mutant strains [21]. Chloramphenicol (3.4 μg/ml) was used for selection of transformants.

Protein extraction, SDS-PAGE, and Western blot analysis. Cells growing exponentially were harvested by centrifugation. The cell pellet was suspended in 10 mM Hepes-KOH, pH 7.6, to adjust the optical density at 730 nm to 10. The cell suspension was mixed with an equal volume of glass beads (Sigma) and cells were disrupted by vortexing to extract soluble proteins [22]. Equal volume (20 μl) of the protein extract was boiled for 3 min in a mixture containing 60 mM Tris-HCl, pH 6.8, 60 mM dithiothreitol, 0.1% SDS, 0.03% bromophenol blue, and 6% sucrose and then loaded onto an 8% polyacrylamide gel in the presence of SDS. Western blot analysis was performed to detect GroEL by using anti-*S. vulcanus* GroEL polyclonal antibodies as probes [22].

DNA mobility shift assays. Exponentially growing cells at 40, 50, or 63˚C in the light

were harvested by centrifugation at room temperature. All of the following procedures were carried out at 4˚C unless stated otherwise. The cells were suspended in 0.24 g of cells/ml of 10 mM Hepes-KOH, pH 7.6, and disrupted by a French press. The resulting cell extract was centrifuged again at 16,000 x g for 20 min. The supernatant fraction was analyzed for DNA binding activity by DNA mobility shift assays. For preparation of a probe, a DNA fragment containing an upstream region of the *groESL1* operon was amplified in the presence of a pair of primers, Tel_ESL1_F and Tel_ESL1_R (Table 1), by PCR. To prepare a double-stranded oligonucleotide competitor containing CIRCE, a pair of sense and anti-sense 28-mer single-stranded oligonucleotides, CIECE_S and CIECE_A (Table 1), were mixed together in an equimolar ratio in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 100 mM NaCl, denatured at 95˚C for 10 min, and then annealed. Assays were performed as in the previous study [23].

Construction of luciferase reporter genes fused with the promoter regions of the *groEL2* gene. DNA fragments of the *groEL2* promoter region were amplified by PCR with a pair of primers containing either the *Bgl*II site or the *Bsi*WI site (Tel_cpn60_-96s_BglII, Tel_cpn60_+38a_BsiWI, Tel_cpn60_-195s_BglII, and Tel_cpn60_-70a_BsiWI. See Table 1). The nucleotide sequences of the promoter regions were confirmed by DNA sequencing. The PCR products were digested with *Bsi*WI and *Bgl*II and cloned into pPT6803-1 digested with the same enzymes to obtain the reporter construct pPgroEL2-luxSpr. The plasmid vector, pPT6803-1, has the promoter-less *luxAB* genes, the neutral site of *Synechocystis* sp. strain PCC 6803 (the downstream region of *ndhB*) and the spectinomycin-resistance (Spr) cassette [24]. The *Synechocystis* sp. PCC 6803 cells were transformed with each construct. Measurements of luminescence from cells harboring luciferase reporter genes were performed as described previously [14].

3. Results

Expression of *groESL1*, *groEL2*, and *hspA* at different temperatures in *T. elongatus*. In order to determine whether *groESL1* and *groEL2* are differentially expressed, we performed Northern blot analysis with *T. elongatus* cells incubated at different temperatures. We determined the level of the *groESL1*, *groEL2*, and *hspA* mRNA in cells incubated at 40, 50, and 63˚C. The normal growth temperature was set to be 50˚C. The *hspA* gene was measured as a control heat shock gene since we have already shown that it is greatly heat-induced in this thermophilic cyanobacterium [23].

As shown in Fig.1A, all these heat shock genes were induced by a shift in temperature to 63˚C. Both *groEL* genes were expressed at 50˚C , while the *hspA* gene was not. The *T. elongatus groEL2* gene reached the peak of its mRNA accumulation earlier than *groESL1* and *hspA*. Thus, *groEL2* may play a role in the early stage of a heat shock response.

Cold shock at 40˚C caused accumulation of *groEL2* mRNA (Fig. 1B). The cold-induction did not show a transient peak within 30 min and sustained a high level for a longer time at 40˚C than at 63˚C. During this time period, *groESL1* were not significantly expressed. The *hspA* mRNA was not detected at all.

This increase in the *groEL2* mRNA level might have been due not only to transcriptional activation but also to stabilization of the mRNA. In order to examine whether increased transcript stability contributed to cold-shock induction of *T. elongatus groEL2*, the decay of the level of the *groE* mRNAs which had accumulated under normal conditions at 50˚C was monitored at 40 and 50˚C, after inhibition of transcription by rifampin (Fig. 1C). Within 15 min after the addition of rifampin, the mRNA level of both *groE* transcripts in cells incubated at 40 and 50˚C reduced to a non-detectable level. In fact, regardless of the incubation temperature, both *groE* transcripts almost disappeared within 5 min in the presence of rifampin. These results indicate that there is no apparent change in

the stability of both *groE* mRNAs in response to temperature change. They also indicate that *groEL2* transcription is greatly activated at 40˚C, whereas the *groESL1* transcription is suppressed.

Analysis of the transcriptional regulatory regions of the *groE* genes. Next, we turned to the mechanisms underlying the differential expression of the two *groE*s, especially at low temperatures. The transcription start sites for *groEs* in thermophilic cyanobacteria had not previously been determined. In order to accurately identify the transcriptional start sites from which the transcription of either the *groESL1* operon or the *groEL2* gene takes place at 40, 50, and 63˚C, primer extension analysis with RNA from *Thermosynechococcus* cells incubated at various temperatures for 15 min was performed. A single and identical 5' end was detected in the *groESL1* mRNA, independent of the incubation temperature. The 5' end was mapped 121 bp upstream of the translational start codons (Fig. 2A). The consensus promoter sequences recognized by the *E. coli* major sigma factor were localized upstream of the transcriptional start site. The CIRCE element, TTAGCACTC-N9-GAGTGCTAA, overlapping the putative –10 promoter sequence was also present, just as in the case of *Synechocystis* and *Synechococcus groESL1* [16, 25]. Although the length of the 5'-untranslated region differed between *Synechocystis*, *Synechococcus*, and *Thermosynechococcus*, the location of CIRCE in relation to the transcription start site (C) of the *groESL1* operons was completely conserved (Fig. 2B). The transcription of *groESL1* from the vegetative promoters overlapping CIRCE was clearly induced at 63˚C (Fig. 2A).

Thus, our results strongly indicate that the transcription of *groEL1* is repressed under normal growth conditions by the interaction of HrcA with CIRCE. As proposed for *Bacillus subtilis* [15], the *groESL1* operon may be de-repressed due to the inactivation of the HrcA protein under heat stress. This explanation was supported by DNA mobility shift

assays. As shown in Fig. 2C, we detected a mobility-shift of a *groESL1* probe during PAGE when the CICRE-containing *groESL1* probe was mixed with the extract of cells incubated at 40˚C or 50˚C for 15 min. There was no shift when the probe was mixed with extract of cells incubated at 63˚C. The specific binding of a protein to CIRCE was confirmed by competition assays with an unlabeled 28-nt DNA fragment containing CIRCE. Previously, we had done similar experiments with cell extracts from *Synechocystis* sp. strain PCC 6803 and *S. elongatus* strain PCC 7942. In spite of great effort, we could not detect any mobility shift with CIRCE-containing DNA fragments [14]. Like the HrcA protein of *Bacillus subtilis* [26], HrcA from these mesophilic cyanobacteria may have a strong tendency to aggregate. There had thus been no direct evidence that CIRCE acts as a protein binding element in cyanobacteria. The HrcA protein from *T. elongatus* may be more stable than HrcA proteins from these mesophillic cyanobacteria.

In contrast to the *groESL1* mRNA, two potential transcriptional start sites for *groEL2* were identified, 73- and 166-bp upstream of its translational start codon as two 5' ends of the *groEL2* mRNAs were detected by the primer extension analysis (Figs. 3A and 3B). The consensus promoter sequences were found to be present upstream of the two sites (Fig. 3C). The question arose whether both of these promoter sequences were functional, or if the 5'-end of the shorter *groEL2* transcript was only the result of degradation of the longer mRNA. We thus performed promoter assays, but in *Synechocystis* sp. strain PCC 6803 instead of *Thermosynechococcus*, since promoter assays in *Synechocystis* are well established by ample reports and since it was much easier to transform the mesophilic cyanobacterium than the thermophilic one. As shown in Fig. 4, both promoters were active and we concluded that both promoters are functional in the transcription of the *Thermosynechococcus groEL2*. The TS2 promoter was also active in *E. coli* (data not shown), which further confirms that the shorter mRNA is not the degradation product of the

longer one. We performed a gel-shift assay in order to identify a protein that binds specifically to an upstream region of *groEL2*. We used several probes of various lengths from +14 to –268 relative to the translational initiation site, and used extracts from various cultures. However, we could not detect any protein binding to the probes (data not shown). These negative results support the idea that the HrcA protein is not involved in the transcriptional regulation of the *groEL2* gene.

Disruption of *groEL2*. The transcriptional activation of the *groEL2* gene indicates a role of this gene under heat and cold stresses. To test whether the absence of GroEL2 would affect the performance of *T. elongatus* under heat and cold stress conditions, the *groEL2* gene was disrupted by double crossover insertion of a chloramphenicol resistance gene cassette into the chromosome (Fig.5A). Correct integration and complete segregation of the mutation in all the copies of the genome was confirmed by PCR (Fig.5B). The results indicate that the gene is dispensable under normal growth conditions at 50˚C. This is the first report of the construction of a cyanobacterial mutant lacking a GroEL.

No difference in growth rates was observed at 50˚C (Fig. 6A). However, the mutant cells stopped growing immediately after a shift to 62˚C (Fig.6B). The mutant was also cold-sensitive as it stopped growing 40 h after a shift to 40˚C (Fig. 6C). The level of GroEL2 was higher at 40˚C in the wild type cells than at 50˚C, while GroEL2 was not detected in the mutant cells incubated at those temperatures, confirming the inactivation of the *groEL2* gene in the mutant (Fig.6D). It was possible to separate GroEL2 from GroEL1 since the electrophoretic movement of the GroEL2 protein during SDS-PAGE was slower than that of the GroEL1 protein, which was confirmed by Western blot analysis using cell extract of an *E. coli* strain which over-expresses either *S. vulcanus* GroEL1 or GroEL2 (data not shown). The amino acid sequences of GroEL1 and GroEL2 of the thermophilic cyanobacterium *S. vulcanus* [12, 17] are the same as those of *T. elongatus* (CyanoBase).

4. Discussion

We have shown that the *groEL2* gene, as well as the *groESL1* operon, is heat-induced in the thermophilic cyanobacterium *T. elongatus*. Cold-shock induces only the *groEL2* gene greatly. Our primer extension analysis and DNA mobility shift assays strongly suggest that while the *groESL1* operon is regulated by the CICRE/HrcA system, the *groEL2* gene is regulated by other mechanisms. The GroEL2 protein is not essential under normal growth conditions, but plays an important role under high and low temperature stresses.

An important finding of the present study is that the GroEL2 protein is required for the bacterial growth at high and low temperatures, although the GroEL1 protein is co-existing at these temperatures. As shown in Fig. 6D, the cellular level of the GroEL2 protein at 40^{o}C is less than the GroEL1 protein. Thus, GroEL1 cannot substitute for GroEL1 under the stress conditions. As described above, the amino acid sequences of GroEL1 and GroEL2 of *S. vulcanus* are the same as those of *T. elongatus*. Thus, as shown in these GroELs [12, 17], we can predict that *T. elongatus* GroEL1 complements the temperature-sensitive phenotype of the *E. coli groEL* mutant, while GroEL2 does not. In total, our results strongly indicate that the *groEL2* paralog in *T. elongatus* has evolved unique properties to protect cells under stresses such as heat and cold stress.

In *E.coli*, expression of the *groESL* operon is suppressed in the cold, and its overproduction, while protective against high temperatures, reduces viability at 4°C [27]. Just like the *E. coli groESL* operon, expression of the *T. elongatus groESL1* was suppressed in the cold, while that of the *groEL2* was induced (Fig. 1B). The *T. elongatus* GroES/GroEL may provide housekeeping chaperone functions like the *E. coli* GroES/GroEL, while GroEL2 may play a specific role to acclimate to cold stress. The

increase in the GroEL2 level does not have a negative effect for the growth of *T. elongatus* at low temperatures.

The *groEL2* genes have undergone much more divergence than the *groEL1* genes in terms of regulatory elements for transcription and the deduced amino acid sequences (see Figure S2 of reference 14). The results of this study on *T. elongatus* indicate that GroEL2 has diversified in terms of gene expression and function to play roles under heat and cold stresses, whereas GroEL1 focuses on housekeeping more, in conjunction with GroES. The GroEL2 diversification may have an important implication in the cyanobacterial adaptation to a hot or cold environment.

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6. References

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7. Figure legends

Fig. 1. Northern blot analyses. (A) Time course for the accumulation of the *groESL1*, *groEL2*, and *hspA* transcripts from *T. elongatus* cells after a temperature shift from 50˚C to 63˚C for 5, 15, and 30 min. 1.5 μg of total RNA was electrophoresed in a denaturing agarose gel. 2.8-kb, 2.3-kb, and 1.3-kb rRNAs on nylon membrane were detected by methylene blue staining, and shown as references for the amount of RNA loaded to each lane. (B) Time course for the accumulation of the *groESL1*, *groEL2*, and *hspA* transcripts from *T. elongatus* cells after a temperature shift from 50˚C to 40˚C for 5, 15, and 30 min. 1.5 μg of total RNA was electrophoresed in a denaturing agarose gel. The mRNA of the constitutively expressed *rnpB* gene was used as a loading reference. (C) Effect of rifampin on the stability of the *groESL1* and *groEL2* mRNA in *T. elongatus*. Cells growing at 50˚C were kept at either the same temperature or shifted to 40˚C immediately after rifampin was added to a concentration of 200 μg/mL (Rif+). As a control, the same volume of methanol, the solvent used to dissolve rifampin, was added instead of the rifampin solution (Rif-). RNA was isolated and analyzed at the time points shown across the top. 2.3 μg of total RNA was electrophoresed in a denaturing agarose gel. 2.8-kb, 2.3-kb, and 1.3-kb rRNAs on nylon membrane were detected by methylene blue staining, and are shown as references for the amount of RNA loaded to each lane.

Fig. 2. Mapping of the 5'-end of the *groESL1* mRNA by primer extension analysis (A), alignment of nucleotide sequences of 5'-upstream regions of various cyanobacterial *groESL1* (B), and DNA mobility shift assays to detect a protein binding to the *groESL1* upstream region (C). Primer extension reactions were performed with total RNA isolated from cells before (lane 2) and after shifting from 50˚C to either 40˚C (lane 1) or 63˚C

(lane 3) for 15 min. 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 genomic DNA containing *groESL1* as a template. The arrow indicates the location of the extended product. In B, all the transcription start sites (+1) in the alignment were deduced by primer extension analysis, not by *in silico* analysis. The pair of arrows and underlined letters indicate the location of the consensus CIRCE inverted repeat sequences and promoter sequences, respectively. The abbreviations for various species, S6803, S7942, and Telon, indicate *Synechocystis* sp. strain PCC 6803, *Synechococcus elongatus* strain PCC 7942, and *Thermosynechococcus elongatus*, respectively. DNA mobility shift assays were performed with 15 fmol of a DIG-labeled probe and cell extract (150 μg protein, indicated as 'protein +') from *T. elongatus* incubated at 40, 50, or 63˚C for 15 min. C and F stand for protein/probe complex, and free probe, respectively. 'CIRCE +' indicates the addition of a competitor DNA fragment (100 pmol) which contains the CIRCE element.

Fig. 3. Mapping of the 5'-end of the *groEL2* mRNAs by primer extension analysis (A and B), and nucleotide sequence of 5'-upstream region of *groEL2* (C) from *T. elongatus*. Primer extension reactions were performed with total RNA isolated from cells before (lane 2) and after shifting from 50 to either 40˚C (lane 1) or 63˚C (lane 3) for 15 min. 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 genomic DNA containing *groEL2* as a template. Promoter sequences are indicated as underlined letters.

Fig. 4. Detection of the *groEL2* promoters by the *luxAB* reporter assays. (A) Constructs of P*groEL2-luxAB* reporter genes. DNA fragments indicated by lines R1 to R3, were fused

to the promoter-less *luxAB* operon in plasmid pPT6803a. Positions are numbered relative to the translational initiation site (+1) of the *groEL2* gene. The transcription start sites deduced by primer extension analysis are shown as TS1 and TS2. Each strain carrying one of the P*groEL2-luxAB* reporter genes was designated as EL2_R1, EL2_R2, and EL2_R3 according to the fused *groEL2* region (A). (B) Levels of luminescence measured from the control *Synechocystis* strain with no *T. elongatus* DNA and strains harboring various P*groEL2-lucAB* reporter genes. Each value shows means and standard deviations of at least three independent experiments.

Fig. 5. Schematic drawing showing the gene organization, restriction sites, *groEL2*-encoding region of the chromosomal site, and the site where a chloramphenicol-resistant gene cassette (Cm) was inserted (A) and PCR analyses using chromosomal DNAs of the wild type (WT) and of the mutant (ΔEL2) of *T. elongatus* as a template and primers specific for the *groEL2* gene in order to verify the disruption in the chromosomal DNAs of the mutant. Lane M, HindIII-cut DNA used as fragment size marker.

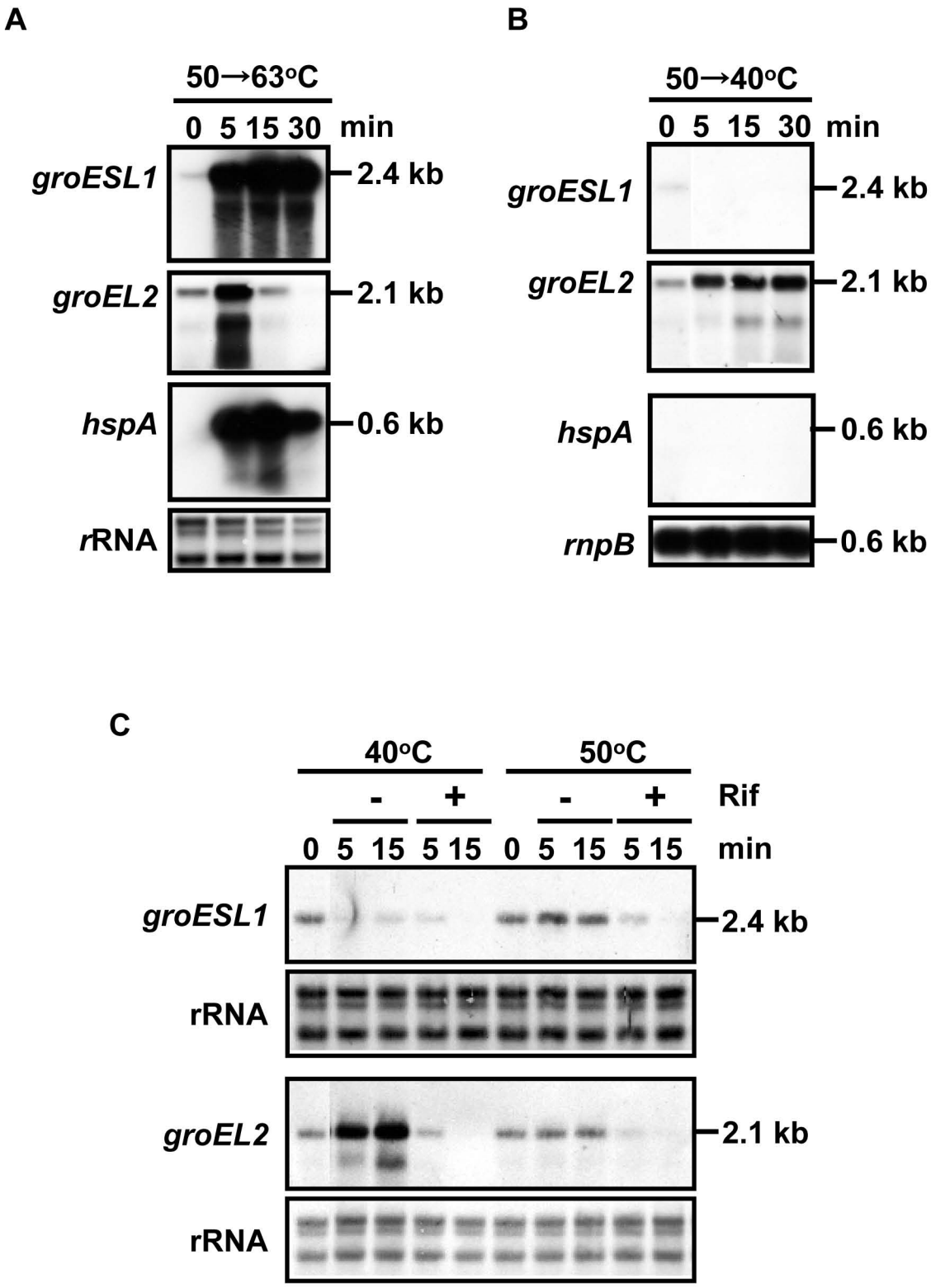
Fig. 6. Growth analyses of the wild type (WT) and the *groEL2* mutant (ΔEL2) of *T. elongatus* at 50˚C (A), 62˚C (B), 40˚C (C) after a shift from 50˚C, and Western blot analysis of GroEL1 and GroEL2 using extracts of equal number of cells before or after a shift to 40˚C for 24 h from 50˚C (D). In the growth curves, each value of the apparent optical density at 730 nm (OD_{730}) of a cyanobacterial culture shows the mean of 3 independent experiments with all the strains. The circle, and square symbols indicate the wild type, and the *groEL2* mutant, respectively.

Table1. Sequences of oligonucleotide primers used for probe preparation.

Primer name	Primer sequence (5' to 3')[a]	Usage[b]
Tel_groES_01	GCGTTTCAACTGTGAAGC	groESL1_N
Tel_groES_02_T7	taatacgactcactatagggCCCACAATGGCCAAGATG	groESL1_N
Tel_cpn60_01	CCAGTCGCATTGACGCAC	groEL2_N
Tel_cpn60_02_T7	taatacgactcactatagggCCCATACCGCCATTAGCG	groEL2_NM
Tel_hspA_06	AGGTGGCCTAAGCTTCATAGTT	hspA_N
Tel_hspA_07	GAAGTCCCTCGAGTGATCCTGTG	hspA_N
Rnase_P3	AGTTAGGGAGGGAGTTGC	RNase P_N
Rnase_P2_T7	taatacgactcactatagggTAAGCCGGGTTCTGTTCC	RNase P_N
Tel_groES_PriEx_1	TTCACAGTTGAAACGCTTAGAGATACG	groESL1_P
Tel_cpn60_PriEx_1	TCTTCATGAAATGCAACTAACTTTGCC	groEL2_P
Tel_cpn60_PriEx_2	TCACAATACTCTGGAGCAGTGTCTTAG	groEL2_P
Tel_cpn60_PriEx_3	TGGCAACACATCAGCAGACTTCTTAGC	groEL2_P
Tel_ESL1_F	TCGCGGTATTGCCACTACGG	groESL1__E
Tel_ESL1_R	GGGAGTGAGAGCATCCTTGG	groESL1_E
CIECE_S	TTAGCACTCGTGAGGGTGGGAGTGCTAA	groESL1_E
CIRCE_A	TTAGCACTCCCACCCTCACGAGTGCTAA	groESL1_E
Tel_cpn60_-96s_BglII	ggagaTCTGCTGATGTGTTGCCAG	groEL2_R
Tel_cpn60_+38a_BsiWI	ttcgtaCGGGATTGAGGAACTGC	groEL2_R
Tel_cpn60_-195s_BglII	tgagatcTCCTTGGCCGCTGCAAG	groEL2_R
Tel_cpn60_-70a_BsiWI	ttcgtaCGCCCATCCTGGCAACAC	groEL2_R
Tel_cpn60_-885_F	GGACGTGGAGAGGCCGTAAG	groEL2_M

a. Lower-case letters and underlined lower-case letters indicate a restriction site and a T7 RNA polymerase promoter sequence, respectively.

b. The last capital letters, N, M, P, E, and R, indicate Northern blot analysis, construction of mutants, primer extension analysis, DNA mobility shift assays, and reporter assays, respectively.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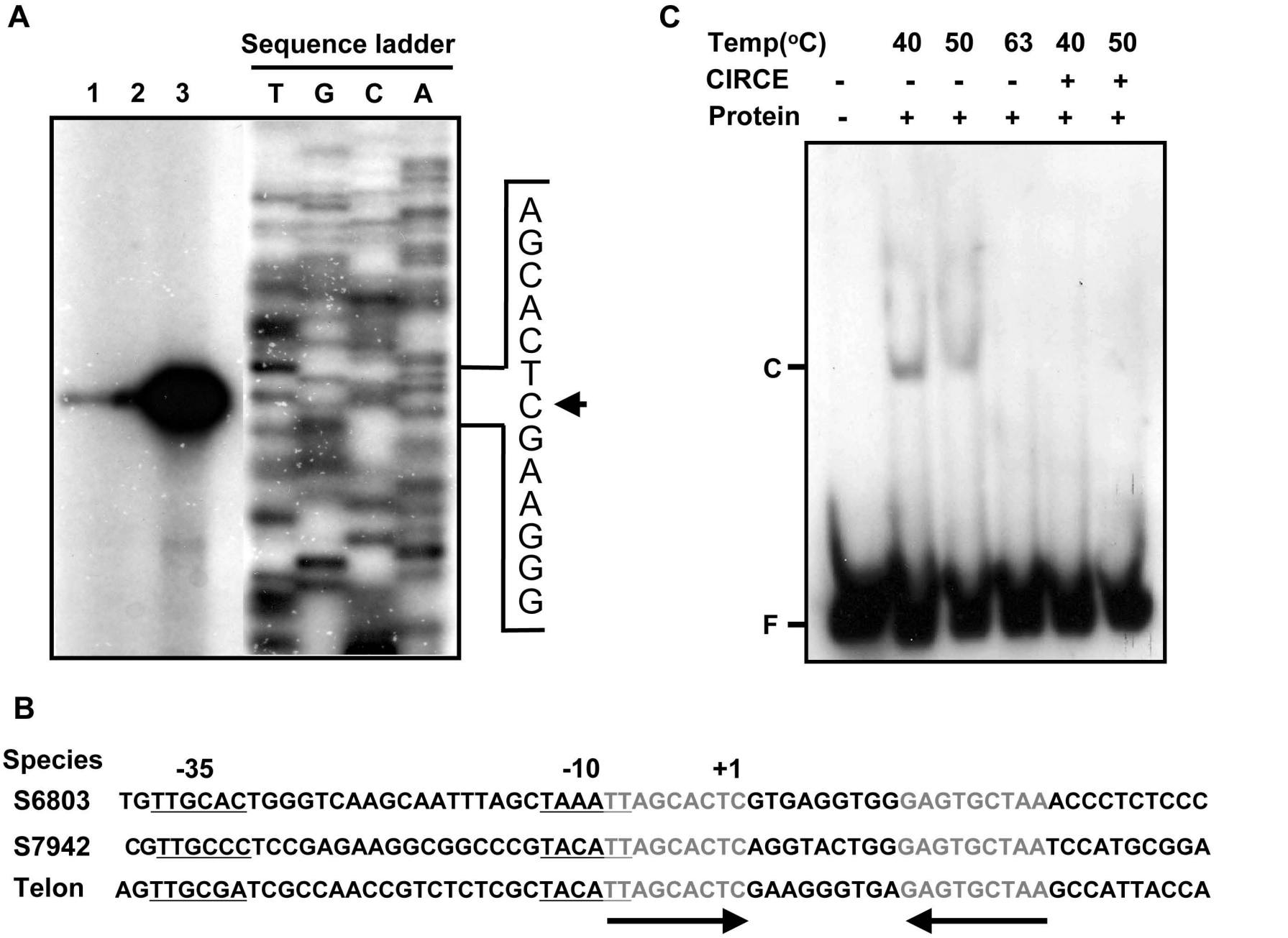
rRNA

Fig.2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C
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CIRCE - - - - + +
Protein - + + + + +
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F
B
Species
-35
-10
+1
S6803 TGTTGCACTGGGTCAAGCAATTTAGCTAAATTAGCACTCGTGAGGTGGGAGTGCTAAACCCTCTCCC
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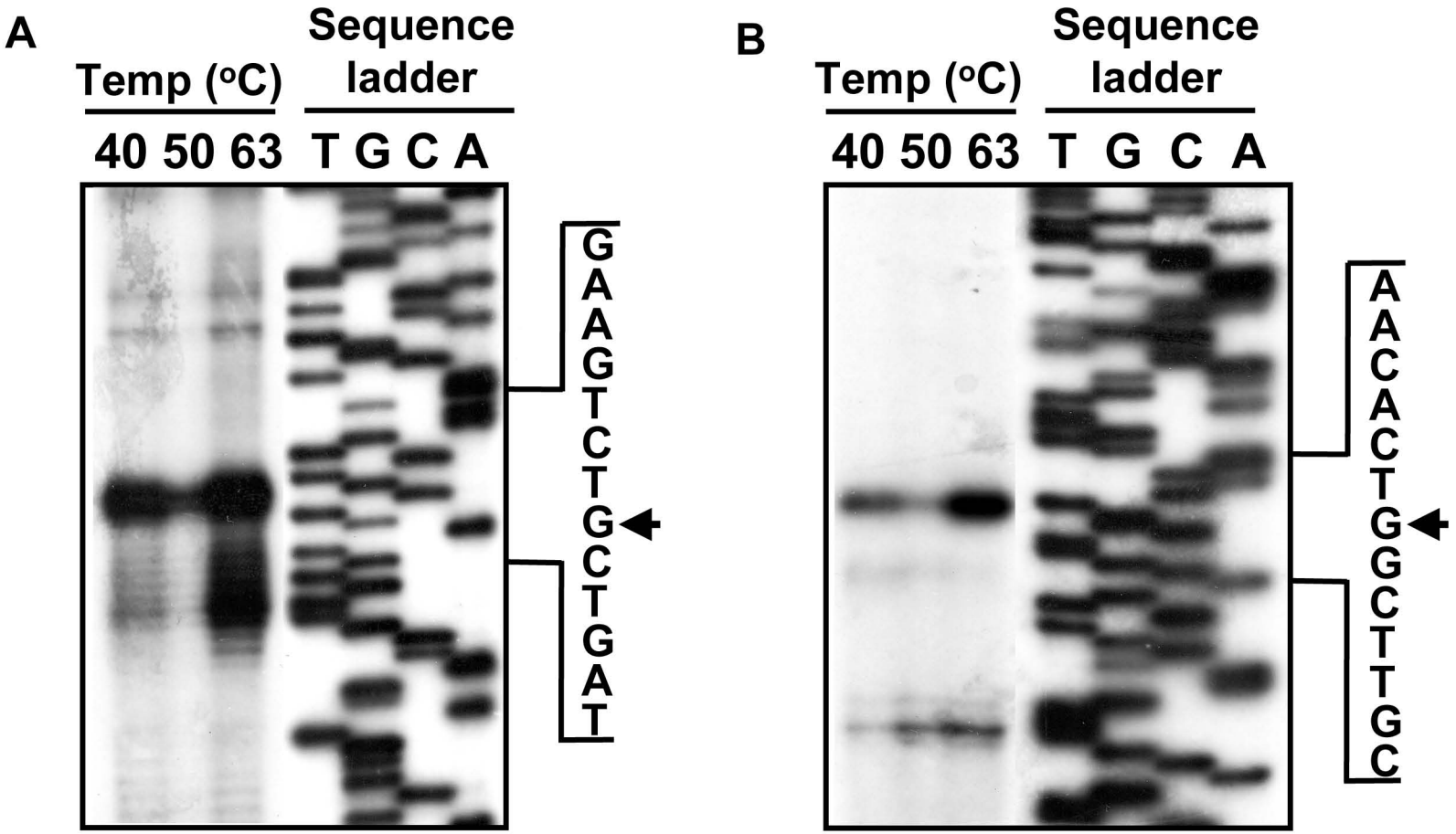

C

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+1

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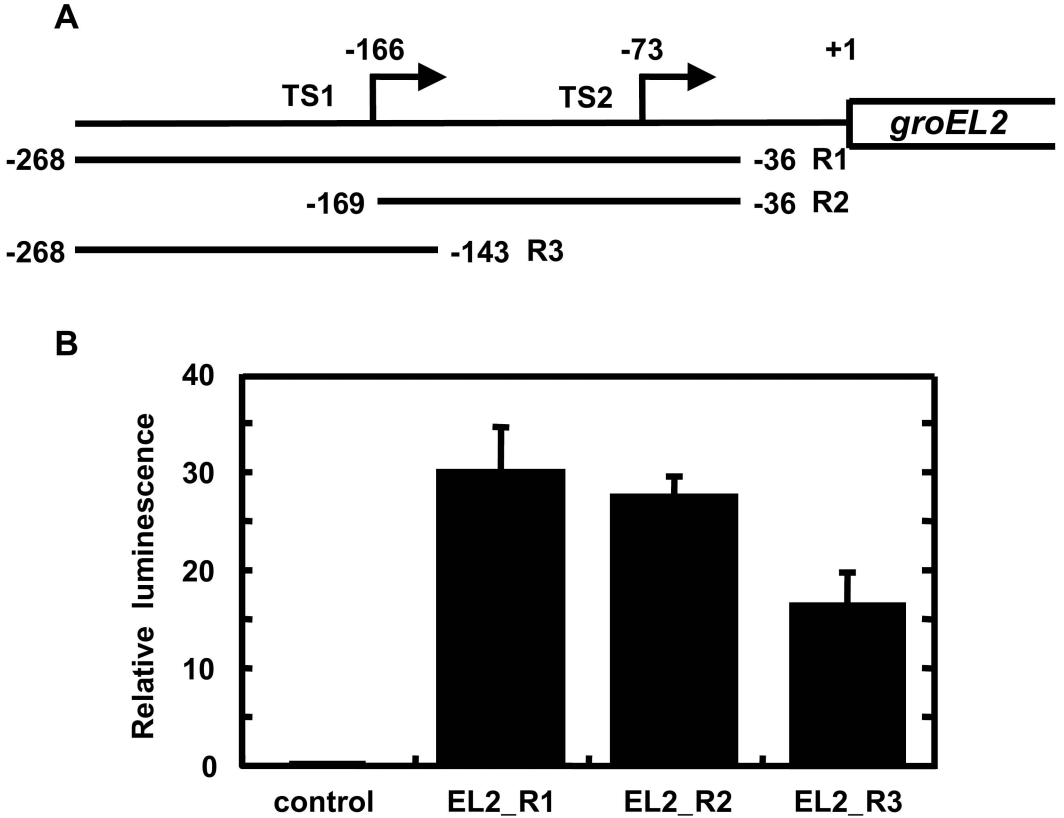
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10
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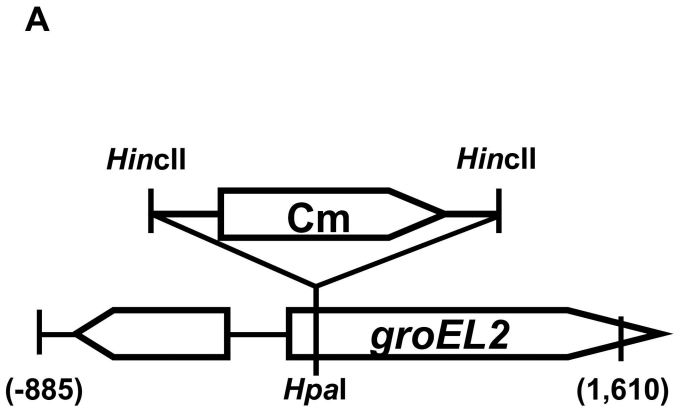
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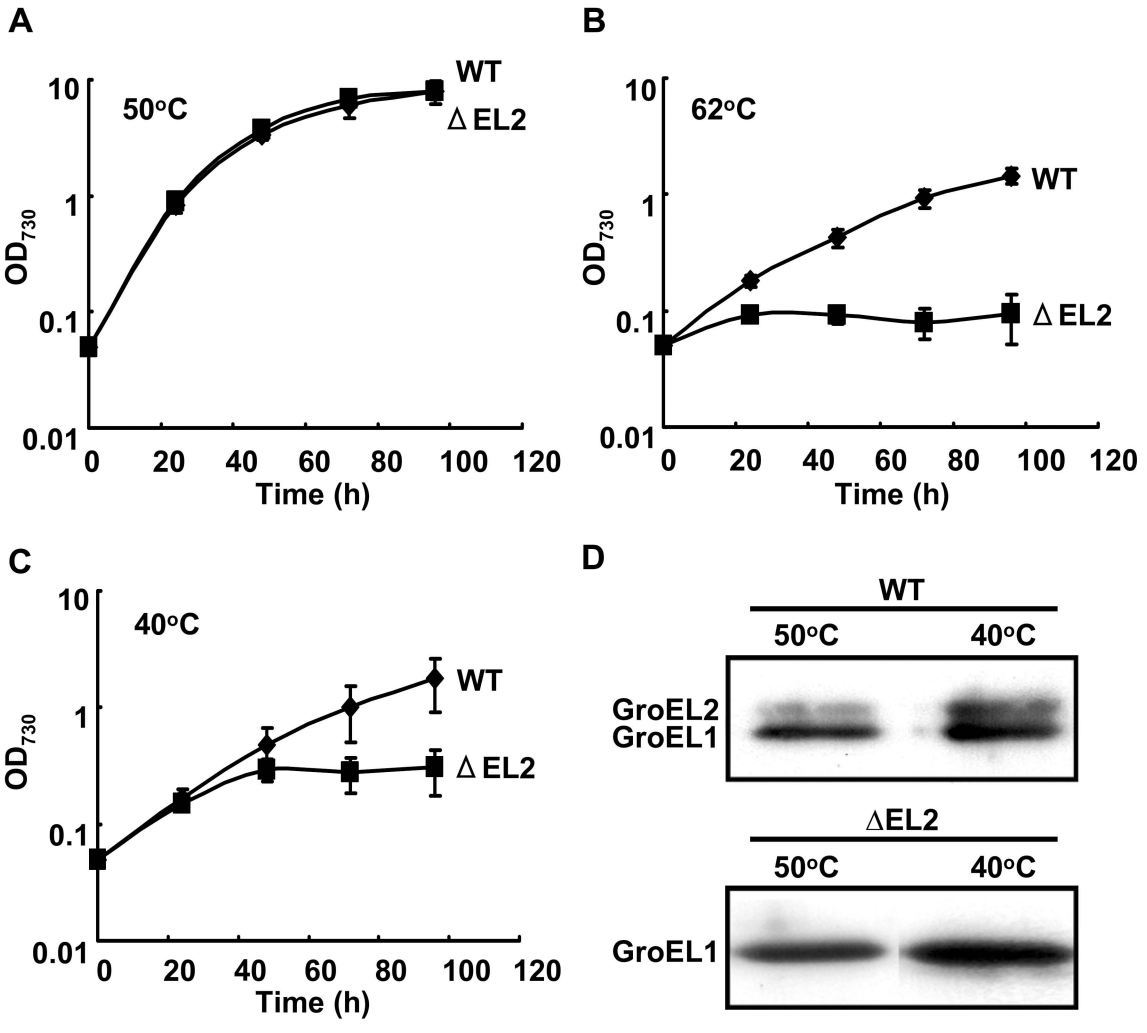
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OD730
Time (h)
D
WT
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40°C
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GroEL1
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40°C
GroEL1