Improving the Productivity of Recombinant Protein in *Escherichia coli* Under Thermal Stress by Coexpressing GroELS Chaperone System

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Here, we demonstrate that the overexpression of the GroELS chaperone system, which assists the folding of intracellular proteins and prevents aggregation of its biological targets, can enhance the thermotolerance of *Escherichia coli* strains and facilitate the production of recombinant protein under thermal stress. The overexpression of GroELS led to an about 2-fold higher growth rate of *E. coli* XL-1 blue than control at 45°C and induced the growth of the strain even at 50°C, although the growth was not sustained in the second-round culture. The effect of GroELS overexpression was also effective on other *E. coli* strains such as JM109, DH5α, and BL21. Finally, we have shown that coexpression of GroELS allows us to produce recombinant protein even at 50°C, a temperature at which the protein production based on *E. coli* is not efficient. This study indicates that the employment of the GroELS overexpression system can expand the range of environmental conditions for *E. coli*.

Keywords: GroELS, thermotolerance, recombinant protein, *Escherichia coli*

Conferring a tolerance to a microbe against unusual environmental conditions such as low temperature and high temperature can expand the applicability of a microbe [6, 7, 22]. For example, developing thermotolerant microorganisms is a crucial event for massive enzyme production for industrial applications [1, 12]. However, improving the tolerance of microbes is not a simple task, since cellular activity is determined by the activities of many intracellular proteins. We may have to engineer a number of proteins involving cellular activity in order to engineer whole-cell activity, which may be hard to be accomplished.

The GroELS chaperone system in *Escherichia coli*, composed of GroEL, a homotetradecameric doubling-ring of 57 kDa subunits, and its co-chaperone GroES, a homoheptameric single ring of 10 kDa subunits, assists the folding of intracellular proteins and prevents the aggregation of its biological targets [2, 3, 20, 21, 26]. In the chaperone system, GroEL, a family of HSP60 chaperones, interacts with up to 50% of intracellular soluble proteins under nonnative conditions such as increased temperature in which spontaneous folding cannot occur [27], whereas it interacts with approximately 10% of newly synthesized polypeptides including some essential proteins for the growth and viability of *E. coli* under normal condition [15]. Several reports have demonstrated that the GroELS, which interacts with a number of intracellular proteins, may be a candidate to control the overall cellular activity [5–7, 16, 19]. It has been shown that the cellular activity under unfavorable conditions of cells can be improved by simply changing the intracellular activity of GroELS. For instance, it has been reported that it is possible to enhance the growth rate of *E. coli* at low temperature by expressing GroELS homologs exhibiting improved activity for protein refolding at low temperature [7, 16, 19]. Desmond *et al.* [6] reported that overexpression of GroELS had improved the tolerance of probiotic strain *Lactobacillus paracasei* against heat stress, salt stress, and organic solvent stress. Moreover, there has been a report that the overexpression of *groELS* genes improved the freeze tolerance of *E. coli* [5].

Here, we report another example demonstrating that the GroELS chaperone system can be used to engineer a microbial activity. We attempted to improve the ability of *E. coli* to grow under thermal stress by overexpressing the GroELS chaperone system. Generally, *E. coli* becomes defective above 37°C, an optimal temperature for its growth. The poor growth of *E. coli* under thermal stress may be related with the decreased activities of many intracellular proteins caused by the heat-induced misfolding of intracellular

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proteins. In this study, we demonstrate that overexpression of the GroELS chaperone system known to assist the folding as well as preventing the misfolding of intracellular proteins under nonnative condition can confer thermotolerance to *E. coli*. In particular, we focused on the effect of GroELS overexpression on the growth ability of *E. coli* under thermal stress, instead of the viability of *E. coli* on which most previous studies have centered [6, 14, 18, 24]. We also demonstrated that the overexpression of GroELS, which enhances the ability of *E. coli* to grow under thermal stress, canly facilitate the production of recombinant protein at an unusually high temperature for *E. coli*.

PCR reagents, T4 DNA ligase, and restriction endonucleases were purchased from Promega (Madison, WI, U.S.A.). The isopropyl β-D-thiogalactopyranoside (IPTG) and arabinose were purchased from Sigma Chemicals (Sigma-Aldrich Corporation, St. Louis, MO, U.S.A.). The host bacterium *Escherichia coli* strain XL1-blue (Stratagene, La Jolla, CA, U.S.A.) was used for plasmid DNA preparation in this study. *E. coli* cells with plasmids were grown aerobically in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, MI, U.S.A.) or on LB agar plate, supplemented with kanamycin (50 μg/ml or 100 μg/ml of ampicillin) for the selection of transformants. *E. coli* BL21(DE3) (F^- *ompT gal dcm lon* $hsdS_B (r_B^- \ m_B^-)$ λ (DE3), JM109 (e14 (McrA-) *recA1 endA1 gyrA96 thi-1 hsdR17* $(r_k^- \ m_k^+)$ *supE44 relA1* (*lac-ProAB*) (F' *traD36 proAB lacI^qZ ΔM15*), XL1 blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F *proAB lacI^qZ M15 Tn10* (*Tet^r*), and DH5α (F' *phi80d lacZ ΔM15* Δ (*lacZYA-argF*) *U169 deoR recA1 endA1 hsdR17*
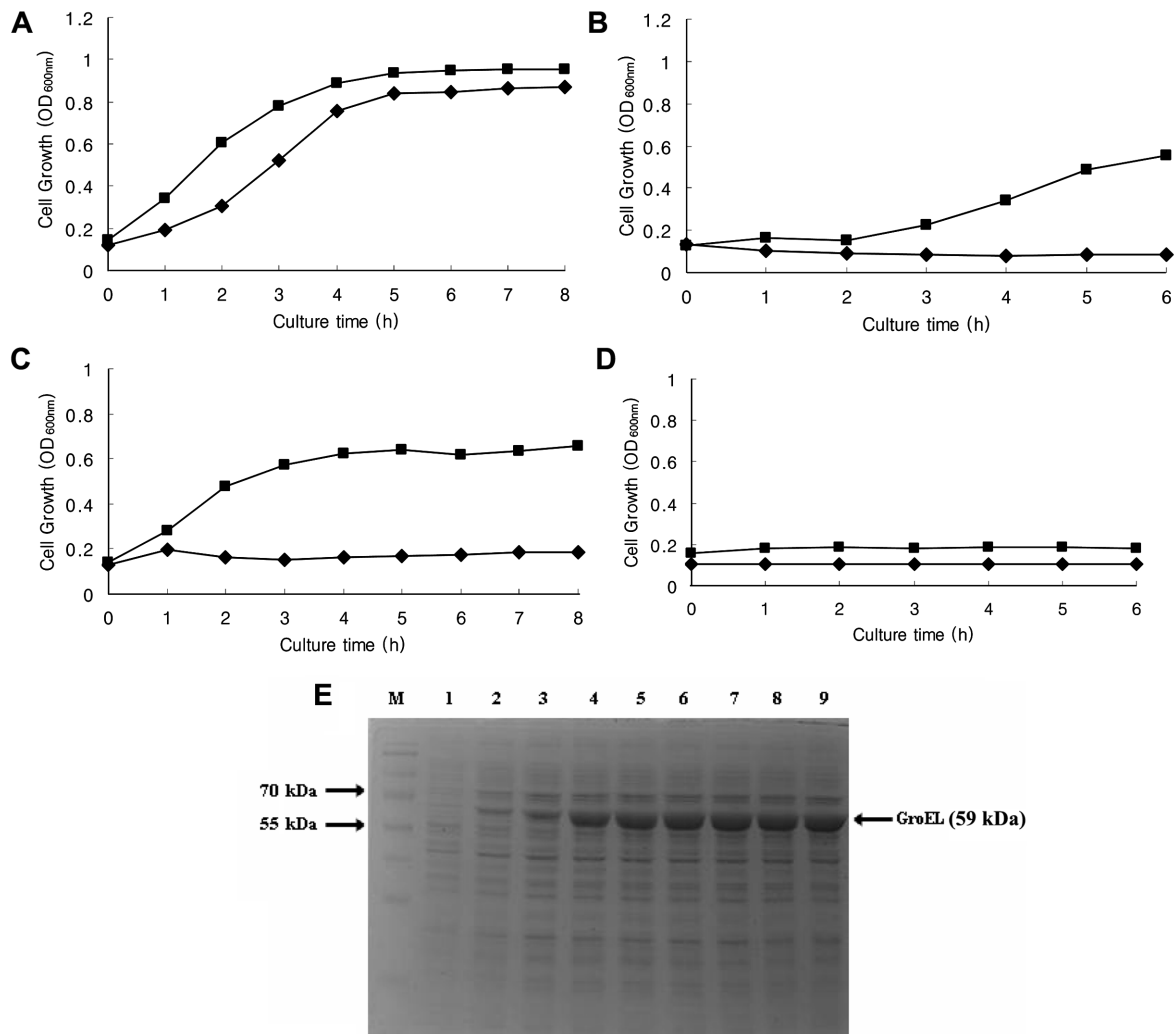

Fig. 1. Effect of overexpression of GroELS on the growth of *E. coli* XL1-blue at 45°C and 50°C.
A. Growth profiles of XL1/pBAD (◆) and XL1/pBAD-GroELS (■) in the first-round culture at 45°C. **B**. Growth profiles of XL1/pBAD (◆) and XL1/pBAD-GroELS (■) in the second-round culture at 45°C. **C**. Growth profiles of XL1/pBAD (◆) and XL1/pBAD-GroELS (■) in the first-round culture at 50°C. **D**. Growth profiles of XL1/pBAD (◆) and XL1/pBAD-GroELS (■) in the second-round culture at 50°C. **E**. SDS-PAGE of the samples for XL1/pBAD-GroELS in the experiment of Fig. 1C. (M: size marker; lanes 1–9: samples at 0–8 h).

$(r_K^- \ m_K^+)$ *phoA supE44 λ-thi-1*) were used as host cells for *groELS* expression. pBAD-HisC from Invitrogen (Invitrogen, Carlsbad, CA, U.S.A.) [10] was used as a negative control for *groELS* expression, and pBAD-GroELS containing the pBR322 replication origin and ampicillin resistance gene was used for the overexpression of GroELS [11]. pET24ma, a derivative of pET24a containing the p15A replication origin and kanamycin resistance gene [17], was used as backbone for the construction of the pET24-GFP.

The DNA manipulations were performed according to the procedures described by Sambrook and Russel [23], and the pGEM-T vector system (Promega Corporation, Madison, WI, U.S.A.) was used for the cloning of PCR products. The PCR reaction (50 μl) contained 50 pmole of primer, template DNA, 1× *Taq* DNA polymerase buffer, 0.025 units of *Taq* DNA polymerase, 2.5 mM deoxyribonucleotide triphosphates, and 1.5 mM $MgCl_2$. Amplification was performed in a DNA thermal cycler (Master Gradient thermal cycler, Eppendorf, Hamburg, Germany) programmed for an initial denaturation (94°C for 1 min) followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C, with an extension at 72°C for 10 min.

pBAD-GroELS was constructed as described earlier [11]. The pET24-GFP plasmid was constructed by PCR-amplified DNA fragment of GFP using the forward primer 5'-GGATCCCGTAAAGGAGAAGAACTTTTC-3' and reverse primer 3'-GAGCTCTTATTATTTGTATAGTTCA-TCCA-5' from pGlnAp2-GFP$_{mut3.1}$ [4]. The PCR-amplified DNA fragment was purified using a PCR clean-up system (QIAquick PCR Purification kit; Qiagen, Valencia, CA, U.S.A.), and ligated into BamHI and SacI sites in the pET24ma vector [17].

To investigate the effect of GroELS overexpression on the thermotolerance of *E. coli*, we first examined the growth of *E. coli* XL1-blue strains harboring pBAD-HisC or pBAD-GroELS at 37, 40, 45, 50, and 55°C. Hereafter, the *E. coli* XL1-blue harboring pBAD-HisC will be referred to as XL1/pBAD and the *E. coli* XL1-blue harboring pBAD-GroELS will be referred to as XL1/pBAD-GroELS. The two strains were grown until the OD_{600} of the cultures reached about 0.6 at 37°C, then diluted to the OD_{600} of 0.1, transferred to the shaker of different temperatures, and their growth was monitored.

At 37°C and 40°C, the growth patterns of XL1/pBAD and XL1/pBAD-GroELS were almost the same (data not shown). At 45°C, the growth rate of XL1/pBAD-GroELS was approximately two times higher than that of XL1/pBAD (Fig. 1A). To investigate whether the effect is sustained, the two strains were diluted to the OD_{600} of 0.1 when they reached their stationary phase and second-round culture was performed. As shown in Fig. 1B, the growth of XL1/pBAD almost halted in the second-round culture. On

the other hand, the growth of XL1/pBAD-GroELS was observed, although the growth rate of XL1/pBAD-GroELS in the second-round culture was decreased by about 3-fold compared with that of the first-round culture shown in Fig. 1A. The growth curves of the two strains at 50°C exhibits that XL1/pBAD-GroELS grew but reached stationary phase at the OD_{600} of 0.6 and XL1/pBAD did not grow (Fig. 1C). When the two strains were diluted to the OD_{600} of 0.1 again and second-round culture was performed, both strains did not show any growth (Fig. 1D). SDS-PAGE patterns of protein samples obtained from first-round culture at 50°C confirm that GroEL was overexpressed in the XL1/pBAD-GroELS (Fig. 1E). We could not detect the overexpression of GroES, probably because of inefficient expression of the second gene in the bicistronic gene expression system. At 55°C, the two strains did not grow at all when the two strains were transferred from 37°C to 55°C (data not shown).

The above results firstly indicate that wild-type *E. coli* XL1-blue becomes defective as the growth temperature is increased. The growth of wild-type strain was temporally sustainable at 45°C, but totally hampered at 50°C and 55°C. Secondly, *E. coli* XL1-blue becomes thermotolerant by overexpressing GroELS in the cytoplasm of the strain. The growth rate of the strain overexpressing GroELS was achieved even at 50°C, although the growth was not sustained and the culture reached the stationary phase earlier than the growth of *E. coli* under normal growth condition owing to thermal stress, suggesting that the overexpression of GroELS may enable us to circumvent the temperature limitation for the growth of *E. coli* XL1-blue.

In our previous study, we have observed that the effect of GroELS activity on the growth of *E. coli* can be varied depending on the genotype of *E. coli* [16]. For example, although we have selected a GroELS variant that enhances the growth rate of *E. coli* at low temperature, the variant was only effective to the DH5α strain, which limited the practical use of the variant. To investigate whether the above GroELS overexpression system that enhances the ability of XL1-blue to grow under thermal stress can be practically employed, we examined the effect of GroELS overexpression on the growth of other *E. coli* strains; that is JM109, DH5α, and BL21(DE3). *E. coli* JM109 and DH5α are the derivatives of *E. coli* K12 and, like *E. coli* XL1-blue, intensively used as host strains in molecular biology. *E. coli* BL21(DE3) is a derivative of *E. coli* B strain, effectively employed for the expression of recombinant proteins. The strains containing pBAD-HisC or pBAD-GroELS were grown until the OD_{600} of the cultures reached about 0.6 at 37°C, diluted to the OD_{600} of 0.1, transferred to the shaker of 50°C, and their growth was monitored. We observed almost the same results as that obtained using XL1-blue (data not shown). All the *E. coli* strains containing

pBAD-GroELS grew, but the growth of the strains containing pBAD-HisC was limited. These results indicate that the effect of the overexpression of GroELS on the growth of *E. coli* under thermal stress is not limited to XL1-blue, but is effective to other *E. coli* strains of different genotypes, which may allow us to employ the thermotolerant *E. coli* system more practically.

The above results suggest that overexperssion of GroELS may permit us to use *E. coli* even at unfavorable temperatures. For example, we may be able to use *E. coli* even at the temperatures that normally *E. coli* cannot be grown, such as 50°C, through overexpressing GroELS. To demonstrate this, we attempted to produce a recombinant protein by using GFP as a model at 50°C. pET-GFP compatible with the pBAD system was transformed to each strain, that is BL21(DE3)/pBAD and BL21(DE3)/pBAD-GroELS, to form BL21(DE3)/pBAD/pET-GFP and BL21(DE3)/pBAD-GroELS/pET-GFP, respectively. The two strains were grown until the OD_{600} of the cultures reached about 0.6 at 37°C, diluted to the OD_{600} of 0.1, transferred to the shaker of 50°C, and induced with 1 mM IPTG after 3 h of growth. Fig. 2A illustrates the growth of each strain at 50°C, showing that the BL21(DE3)/pBAD-GroELS/pET-GFP grew for 2 h, but BL21(DE3)/pBAD/pET-GFP did not show any growth. These results suggest that the expression of GroELS induced the growth of the strain under thermal

stress, which coincides with the above results. The expression of GFP in the two strains after induction at different time intervals was analyzed by SDS-PAGE. In Fig. 2B, lanes 1, 3, 5, 7 represent the samples of BL21(DE3)/pBAD/pET-GFP at 3, 4, 5, 6 h in Fig. 2A, and lanes 2, 4, 6, 8 represent the samples of BL21 (DE3)/pBAD-GroELS/pET-GFP at 3, 4, 5, 6 h in Fig. 2A. As shown in the data, the total amount of protein produced from BL21(DE3)/pBAD-GroELS/pET-GFP (lanes 2, 4, 6, 8) was much higher than that from BL21(DE3)/pBAD/pET-GFP (lanes 1, 3, 5, 7), which correlates with the cellular concentration shown in Fig. 2A. The data also illustrate the obvious expression of recombinant GFP in the BL21(DE3)/pBAD-GroELS/pET-GFP, whereas there was no expression in BL21(DE3)/pBAD/pET-GFP. In this experiment, we did not evaluate the amount of soluble proteins or insoluble proteins since our interest was only in the investigation of protein production. We presume that the GFP produced in BL21(DE3)/pBAD-GroELS/pET-GFP may be misfolded under thermal stress because the fluorescence of the strain was very weak (data not shown). These results indicate that the *E. coli* harboring overexpressed GroELS in the cell is more active compared with the one without overexpressed GroELS under thermal stress, which enables us to produce recombinant protein at 50°C, the temperature at which the production of recombinant protein production is not feasible by using a normal *E. coli* strain.

In this paper, we have demonstrated that the overexpression of GroELS confers thermotolerance to *E. coli*. In fact, there have been some reports showing that overexpression of molecular chaperones such as GroELS can enhance the thermotolerance of microorganisms, which may be caused by the folding or refolding activity of the chaperone proteins to misfolded cellular proteins under thermal stress [6, 24]. Our study may also be one of the examples to show that whole microbial activity under thermal stress can be improved by increasing the intracellular activity of molecular chaperones. However, whereas most of the previous reports have just focused on the effect of chaperone activity on the viability of microorganisms under heat shock, our study is unique in that we have shown that even the growth of *E. coli* at increased temperature can be achieved by GroELS overexpression. Moreover, we have shown that the production of recombinant protein is possible at an unusually high temperature for *E. coli* by coexpressing the GroELS chaperone system with target protein. This indicates that the employment of the GroELS overexpression system can expand the applicability of *E. coli*, a strain extensively employed in various fields of biotechnology and bioindustry but its utility has been limited within a range of optimal environmental conditions for *E. coli*.

The misfolding of intracellular proteins is recognized as a main factor to make microorganisms inactive under thermal stress [8, 9, 13, 14, 18], although other factors
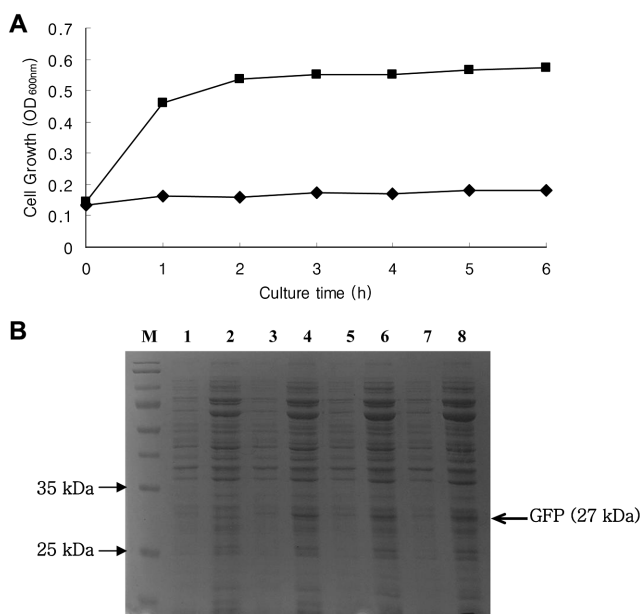

Fig. 2. Effect of overexpression of GroELS on the production of recombinant GFP at 50°C.
One mM IPTG was added to induce GFP at 3 h. **A**. Growth profiles of BL21(DE3)/pBAD/pET-GFP (■) and BL21 (DE3)/pBAD-GroELS/pET-GFP (◆). **B**. SDS-PAGE of the samples for the experiment of Fig. 1A (M: size marker; lanes 1, 3, 5, 7: samples of BL21 (DE3)/pBAD/pET-GFP at 3, 4, 5, 6 h; lanes 2, 4, 6, 8: samples of BL21 (DE3)/pBAD-GroELS/pET-GFP at 3, 4, 5, 6 h).

such as membrane fluidity are also involved in the thermotolerance of microorganisms [25]. Most organisms have defense mechanisms against protein aggregation caused by stress such as heat, and a chaperone system such as GroEL-GroES and DnaK-DnaJ-GrpE plays an important role in the defense system [3, 18, 26]. Many studies have been carried out to understand the interaction of the chaperones with intracellular proteins under normal or thermal stresses, showing that they not only directly interact with a number of intracellular proteins but also affect the some transcriptional networks under stress condition [13, 14, 18]. For example, the GroEL-GroES complex forms an enclosed environment for the correct folding of approximately 50% of intracellular proteins under conditions of cellular stress [27], and the GroEL protein regulates the transcriptional factor σ^{32} [9]. DnaK prevents the aggregation of the intracellular thermolabile proteins with high molecular weights, which confers thermotolerance to *E. coli*, and it is also involved in the regulation of intracellular level σ^{32} [18]. We expect that combination of a few chaperone systems involving defense mechanisms under heat may enable us to achieve more thermotolerable *E. coli*.

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