Overexpression of the *Bacillus licheniformis* GroES enhances thermotolerance of *Bacillus subtilis* WB600

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**ABSTRACT**

Use of thermotolerant strains offers the advantage of conducting production process at elevated temperatures, thereby improving the catalytic efficiency and reducing the energy consumption and the production costs. In the present study, thermotolerance gene *groES* from the thermophilic strain *Bacillus licheniformis* B186 was implanted into *B. subtilis* WB600 to improve its thermotolerance. The engineered strain WB600-pHY-groES displayed reduced doubling time as well as increased specific growth rates and cell viability compared with the control. Our results suggested that introducing heat shock proteins (HSPs) from thermophiles into *B. subtilis* could be an effective approach to enhance its thermotolerance, thus facilitating the development of multichaperone expression systems for constructing more thermotolerant commercial *B. subtilis* strains. To the best of our knowledge, this is the first report on improving the thermotolerance of *B. subtilis* by overexpression of HSPs.

**Abbreviations:** HSPs: heat shock proteins; GRAS: generally regarded as safe; LB: Luria-Bertani; ORF: open reading frame; PCR: polymerase chain reaction; OD600: optical density at 600 nm; qRT-PCR: quantitative reverse transcription PCR; SPSS: Statistical Package for Social Scientists

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**Introduction**

*Bacillus subtilis*, a rod-shaped, Gram-positive and spore-forming bacterium thriving in the soil, has been widely used in food [1], feed [2], pharmaceutical [3], chemical [4,5] and biotechnological industries [6]. For example, *B. subtilis* is used as a host for the production of commercial enzymes, pharmaceutical proteins, biofuels and fine chemicals due to its superior fermentation properties, generally regarded as safe (GRAS) status, high-level protein secretion capability (20–25 g/L), simplified downstream processes [7], as well as other desirable features including the absence of significant codon bias and ease of genetic manipulation [6].

During the fermentation processes, *B. subtilis* constantly faces a variety of environmental stresses, resulting in decreased production capacity. Therefore, improving its cellular robustness to various stresses is of great importance for efficient industrial production of biomolecules, especially enzymes, biochemicals and biofuels [8]. Among the desirable traits of *B. subtilis* for industrial processes, thermotolerance is crucial for minimising the risk of microbial contamination, reducing cooling and product recovery costs [9], and for shortening the fermentation period [10]. To date, many strategies have been developed to obtain super strains exhibiting resistance to elevated temperatures, such as isolation and selection of strains from nature, mutagenesis, protoplast fusion as well as adaptive laboratory evolution. Besides, heat shock proteins (HSPs) are also considered essential components of microbial heat shock responses in that they protect cytoplasmic proteins from irrevocable misfolding, denaturation and aggregation caused by exogenous or biotic stresses. There are two major groups of HSPs, the 70-kDa DnaK family and 60-kDa GroE family [11]. Chaperonins GroES and GroEL from the latter family are the extensively studied HSPs which play an important role in the thermotolerance of *B. subtilis* [12]. Structural and biochemical studies have
demonstrated that the co-chaperone GroES acts with its partner, GroEL, as a two-stroke ATP-regulated folding machine. In addition to preventing aggregation, the GroES-GroEL complex can also provide an enclosed environment for the correct folding of proteins under both normal growth and stressful conditions [11,13]. Therefore, overexpression of HSPs has been used as a promising technique to improve the thermotolerance of Saccharomyces cerevisiae [8], Lactococcus lactis [14–17], Bifidobacterium longum [18], Escherichia coli [19,20] and Anabaena sp. [21]. Although various aspects of the response of HSPs to thermal stress have been extensively studied in Bacillus species, their role in the improvement of thermotolerance is not well documented for B. subtilis.

Previously, the thermotolerance mechanism of the thermophilic strain B. licheniformis B186 was investigated by Dong et al. using tandem mass tag-based quantitative proteomics, and the groES gene encoding a 10 kDa chaperonin was implicated in its thermotolerance [22]. In the present study, the groES gene from B. licheniformis B186 was cloned and heterologously overexpressed in B. subtilis WB600 to enhance its thermotolerance. The effects of groES overexpression on the cell growth and the viability of the engineered strain were then investigated. Our results showed that groES overexpression in B. subtilis WB600 significantly improved its thermotolerance, highlighting the utility of the groES gene for engineering efficient and robust industrial strains.

Materials and methods

Plasmids, primers, bacterial strains and growth conditions

Plasmid pHY-WZX [23] was used for gene cloning and expression. E. coli JM109 and B. subtilis WB600 stocked in our lab served as the hosts for gene cloning and expression, respectively. B. licheniformis B186 with an optimum growth temperature of 50 °C [22] was purchased from CICIM-CU (Jiangnan University, China; http://cicim-cu.jiangnan.edu.cn). All the strains were cultured in Luria–Bertani (LB) medium (yeast extract 5 g/L, tryptone 10 g/L, NaCl 10 g/L). If necessary, 10 µg/mL kanamycin was added to the medium.

Chemicals and reagents

Pyrobest™ DNA polymerase, T₄ DNA ligase and restriction enzymes were purchased from TaKaRa Bio Inc. (Dalian, China). OMEGA Gel Extraction Kit and EZNA™ Plasmid Mini Kit were supplied by OMEGA Bio-Tek (Norcross, GA, USA). EZ-10 Spin Column Genomic DNA Isolation Kit and ampicillin were the products of Bio Basic Inc. (Toronto, Canada) and Sangon Biotech Co., Ltd. (Shanghai, China), respectively. Yeast extract and tryptone were bought from OXOID (Basingstoke, England). All other chemicals used in this study were of analytical grade.

Cloning of groES gene from B. licheniformis B186

Genomic DNA of B. licheniformis B186 was isolated using EZ-10 Spin Column Genomic DNA Isolation Kit according to the instruction manual. The primers designed based on the published genome sequence of B. licheniformis ATCC 14580 (GenBank accession No. Q65MZ9.1) were as follows: groES1 (5’-TGCTCTAGTTTTAAAGCCATTAGGTGATCGC-3’) and groES2 (5’-GGGTAGCCGATAACAGCTAAAAATGTCGC-3’), where the restriction site of XbaI is underlined. The open reading frame (ORF) of the groES gene was then amplified by polymerase chain reaction (PCR) using genomic DNA from B. licheniformis B186 as a template and the primers groES1 and groES2. Amplification was conducted under the following programme: initial denaturation step at 95 °C for 5 min; 30 cycles of denaturation (96 °C for 30 s), annealing (58 °C for 30 s) and extension (72 °C for 1 min); and a final extension step at 72 °C for 10 min (Techne TC-512 thermocycler; Techne Co., Staffordshire, UK). The obtained PCR product was analysed by electrophoresis and purified by TaKaRa MiniBEST DNA Fragment Purification Kit Ver.4.0 (Dalian, China).

Construction of recombinant bacteria

The purified groES PCR fragment was cut by XbaI and ligated with XbaI- and Smal-digested plasmid pHY-WZX, generating the recombinant plasmid pHY-groES. This recombinant plasmid was subsequently transformed into E. coli JM109 competent cells and positive clones were screened on LB plates supplemented with 10 µg/mL kanamycin. After the correctness of plasmid construction was confirmed by restriction analysis and DNA sequencing (Sangon Biotech Co., Ltd., Shanghai, China), the recombinant plasmid pHY-groES and the corresponding control plasmid pHY-WZX were transformed into B. subtilis WB600 competent cells. Recombinant B. subtilis WB600 strains harbouring plasmid pHY-groES (designated WB600-pHY-groES) or pHY-WZX (control, designated WB600-pHY-WZX) were confirmed by colony PCR and plasmid DNA extraction.
Determination of cell viability and cell concentration

Single colonies of B. subtilis WB600-pHY-WZX and WB600-pHY-groES were grown at 37°C for 6 h in LB medium supplemented with kanamycin. To investigate the thermotolerance of B. subtilis WB600, the two cultures were then diluted to an optical density at 600 nm (OD600) of 1.0 and cultured at 37, 42, 50 and 55°C for 1 h. After 10-fold serial dilutions, each of these cultures was spread on LB-kanamycin plates and then aerobically incubated at 37°C for 12 h to observe the growth of the bacteria.

To monitor the growth of B. subtilis WB600 under long-term stress conditions, single colonies of B. subtilis WB600-pHY-groES and WB600-pHY-WZX were inoculated into 250 mL-Erlenmeyer flasks containing 25 mL LB media, and cultured at 37°C and 200 r/min for 12 h. An aliquot of the resulting culture was inoculated in 250 mL shake flasks containing 50 mL LB medium, starting with an OD600 of 0.1 for further cultivation at 37, 42, 50 and 55°C for 12 h, and the cell samples were taken at 2 h-intervals to measure OD600. Doubling time was computed by the online tool (http://www.doubling-time.com/compute.php), according to the duration time, and the initial and final cell concentrations. All data are mean values with standard deviation (±SD) from three independent experiments.

Quantitative real-time PCR

Total RNA from exponentially growing (OD600 of 0.8–1.0) B. subtilis strains was isolated with the RNAprep pure Plant Kit (Tiangen Biotech (Beijing) Co. Ltd., Beijing, China) and then used for cDNA synthesis according to the HiFiScript gDNA Removal cDNA Synthesis Kit (CWBio Co. Ltd., Beijing, China). Primer pairs (groES-F: 5'-TCGTACTCTCTGACTCCGCAA-3' and groES-R: 5'-TGATGCCGGTCGCGTTTT-3') were designed and synthesised to amplify 123-bp regions of groES for quantitative reverse transcription PCR (qRT-PCR). Real-Time PCR was carried out in a 20 μL (total volume) mixture containing 9 μL of SYBR® Premix Ex Taq™ II (2.5×), 0.5 μL each of the forward and the reverse primer (10 μmol/L) and 1 μL of the cDNA sample. The detection was performed as follows: incubation at 95°C for 10 s, 33 cycles at 95°C for 5 s, 51°C for 15 s (Step One System, ABI, USA). The transcriptional level was normalized by the ΔΔCt method [24], with the house-keeping 16S ribosomal RNA as the reference gene for the calculations.

Statistical analysis

Statistical Package for Social Scientists (SPSS) version 19.0 (IBM, Armonk, New York, USA) was used to investigate statistical differences. Values of P < 0.05 were considered significant.

Results and discussion

Overexpression of groES in B. subtilis WB600

In nature, thermophiles show superior robustness under harsh conditions, possibly due to their well-adapted stress-response genes during long-term natural evolution [25], such as molecular chaperones [22]. Overexpression of molecular chaperones, such as HSPs, has been considered as one of the most promising approaches for improving microbial strain tolerance to a variety of stresses, including heat shock [8,15]. In this study, to enhance the thermotolerance of B. subtilis WB600, the groES gene from the thermophilic strain B. licheniformis B186 was introduced into this strain. As described above, an approximately 300-bp PCR product, consistent with the theoretical length of groES (285 bp), was agarose gel-purified and used to construct the expression plasmid pHY-groES, followed by confirmation via sequencing. The 285-bp ORF of groES is predicted to encode 94 amino acids with a theoretical molecular weight of 10.1 kDa. Plasmids pHY-WZX and pHY-groES were then respectively transformed into B. subtilis WB600, generating corresponding recombinant strains WB600-pHY-WZX and WB600-pHY-groES. In the exponential phase of B. subtilis WB600-pHY-groES, the transcriptional levels of
groES were 7.4-fold higher than those in WB600-pHY-WZX as determined by qRT-PCR (Figure 1), suggesting that groES was successfully overexpressed in B. subtilis WB600-pHY-groES.

Thermotolerance of groES-overexpressing B. subtilis WB600-pHY-groES

The growth behaviour of the recombinant and control strains was investigated in LB liquid medium supplemented with kanamycin by exposure to 37°C, 42°C, 50°C and 55°C for 1 h, followed by incubation at 37°C for 12 h. As shown in Figure 2, the cell viability dramatically decreased with increasing temperature, and the recombinant strain B. subtilis WB600-pHY-groES showed relatively higher cell viability than the control cultured at the same temperature.

The experimental analysis of thermotolerance was extended to a study of long-term growth at the aforementioned four temperatures with monitoring at 2-h intervals. Samples were analysed for OD600 and doubling time and the results are summarised in Figure 3 and Table 1, respectively. Variations were observed in both groES-overproducing and control strains. When cultured at the same temperature, the specific growth rate of B. subtilis WB600-pHY-groES was higher than that of the control, and their final cell densities were similar except for cultivation at 37°C. Furthermore, the fermentation periods for both strains decreased with increasing temperature, and the fermentation period of B. subtilis WB600-pHY-groES was shorter than or similar to that of the parent strain (Figure 3). As shown in Table 1, the doubling time of the engineered strain was also shorter than that of the control strain. These results demonstrated that overexpression of groES had a positive effect on the thermotolerance of B. subtilis WB6000.

The misfolding of intracellular proteins is considered to be a main factor making microorganisms inactive under thermal stress, although other factors such as membrane fluidity also affect their thermotolerance [26]. There have been many reports showing that overexpression of HSPs such as GroESL can enhance microbial thermotolerance, which may be due to the folding or refolding activity of the HSPs towards misfolded cellular proteins under thermal stress [13]. Our study may also be one of the examples to show that enhanced microbial thermotolerance can be achieved by the overexpression of HSPs. Besides enhancing the viability of microorganisms under heat shock, GroESL overexpression can also improve diverse types of stress-tolerance of different microbes, including antibiotic resistance [27], stresses of solvents, salinity, drying, etc. [19,21,28,29], thus expanding the applicability of industrial microorganisms, such as B. subtilis WB600.

However, although overproduction of the groES gene improved the thermotolerance of B. subtilis WB6000, the final cell concentration decreased with increased temperature (Figure 3). Further studies are therefore required to increase the final cell density of this thermotolerant strain. As groES genes from extremophiles are superior to those from mesophilic bacteria and they perform quite differently in different hosts [19], the relation and compatibility between the host and extremophilic bacteria must be considered for selecting the proper groES to engineer microbial robustness. Besides, heat shock response usually involves a number of proteins, such as HrcA, DnaK, DnaJ, HtpG, FtsH and GroEL chaperone proteins, and they usually cooperate with each other [12,18,22,30].
Therefore, overexpression of an entire battery of these thermal stress-related proteins may give better cellular survival at elevated temperatures, since it would more closely mimic the heat adaptation response of *B. subtilis*.

**Conclusions**

In the present study, to construct a thermotolerant *B. subtilis* strains, the *groES* gene was cloned from *B. licheniformis* B186 and overexpressed in *B. subtilis* WB600. Our results suggested that overexpression of *groES* showed the potential to improve the capacity of *B. subtilis* to withstand thermal stress conditions, reinforcing the notion that overexpression of HSPs in *B. subtilis* plays an important role in improving the tolerance of cells to heat stress and thus the cell viability. Knowledge gained from this work will also facilitate the development of multichaperone overexpression systems to generate more robust commercial *B. subtilis* strains for the food industry.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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