Comparison of cellular stress levels and green-fluorescent-protein expression in several *Escherichia coli* strains

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Constructs comprising stress-gene promoter elements from *rpoH* (Sigma 32), *clpB* or *dnaK* linked to a green-fluorescent-protein (GFP) expression vector were previously used as non-invasive ‘stress probes’ in *Escherichia coli*. We compared cellular stress responses in four *E. coli* strains: production hosts JM105 and BL21, and cloning hosts HB101 and TOP10. When GFP was also used as a model for foreign protein production, we generally observed that the level of expression was inversely proportional to the level of cellular stress. JM105 showed the highest cellular stress level and very low GFP expression, while BL21 exhibited the lowest cellular stress level and the highest GFP expression, in both normal and heat-shock stress environments.

Introduction

Cells undergo many changes when exposed to chemical or physical stresses, including alterations in gene expression and protein stability. For example, when bacterial cells are exposed to high temperature, heat-shock proteins are transcriptionally up-regulated by transcription factor σ^32^ [1]. These heat-shock proteins are evolutionarily conserved, and may play important roles in folding, assembly, degradation and translocation of proteins under both stress and normal growth conditions [2]. Heat-shock proteins include transcription factors, chaperones, proteases and other proteins that confer a survival advantage on the stressed organism, particularly when the stresses are at sub-inhibitory levels [1]. Heat-shock proteins can also be involved in degradation of foreign proteins in stress and non-stress conditions [3]. Therefore, we hypothesized that the ability of a microorganism to express foreign proteins might be affected by the level of cellular stress [4].

We have developed three ‘stress probe’ plasmids to non-invasively monitor cellular stress responses [5]. Green fluorescent protein (GFP) is used as a reporter for cellular stress response due to its special features, such as no requirement for ATP or other cofactors for fluorescence and the possibility of non-invasive visualization of fluorescence from outside of cells [6]. Cellular stress is detected by fusing the promoter element of a heat-shock stress gene, either transcription factor σ^32^ [7], protease subunit ClpB [8] or chaperone DnaK [9], to the reporter gene *gfpuv*, a UV-optimized variant [10]. This method obviates the need for centrifugation, cell lysis, pH adjustment and subsequent kinetic enzyme activity measurements.

In the present work, using the three stress probes we investigated cellular stress responses in four *Escherichia coli* strains: production hosts JM105 and BL21, and cloning hosts HB101 and TOP10. GFP was also employed as a model foreign protein to investigate more easily the expression capability of each *E. coli* strain.

Materials and methods

Plasmids and strains

Three stress probe plasmids, pGFPuv-Sigma (gfpuv^σ32^; Clontech, Palo Alto, CA, U.S.A.) under the promoter of heat-shock transcription factor σ^32^, pGFPuv-ClpB (gfpuv^clpB^ under the promoter of protease subunit ClpB) and pGFPuv-DnaK (gfpuv^dnaK^ under the promoter of chaperone DnaK), were used to monitor cellular stress levels in different *E. coli* strains (see Figure 1) [5]. The plasmid pBR-GFPuv, which does not have a promoter element for the gfpuv gene, was used as a negative control plasmid for stress response. The plasmid pTH-GFPuv, containing the gfpuv gene under trc promoter and based on a parent plasmid pTrcHis (Invitrogen, Carlsbad, CA, U.S.A.), was used to express GFP as a model foreign protein. *E. coli* JM105 [supE endA sbcB15 hsdRs4 rpsL thi Δ(lac-proAB) F [traD36 proAB- lacI q lacZAM15]) [11], BL21 [F ompT hsdS 8 (r5 m8) gal dcm] [14], HB101 [supE44 hsdS20 (r5 m8) recA13 ara-14 proA2 lacY1 galK2 rpsL20 syl-5 mhl-1] [12], and TOP10 [F mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZ]

Key words: BL21, HB101, JM105, stress probe, stress response, TOP10.

Abbreviations used: GFP, green fluorescent protein; IPTG, isopropyl-β-D-thiogalactoside; SFI, specific fluorescence intensity; SDS/GPAGE, SDS/gelatin PAGE.

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Culture media and growth conditions

To monitor cellular stress responses, recombinant *E. coli* strains harbouring each stress probe plasmid or the control plasmid were grown at 30 °C in 50 ml of LB medium [5 g/l yeast extract (Sigma, St. Louis, MO, U.S.A.), 10 g/l tryptone (Sigma) and 10 g/l NaCl] containing 50 μg/ml ampicillin (Sigma) using 250 ml flasks. These cultures were inoculated (1%, v/v) from 30 °C overnight cultures in the same medium. Heat shock was induced after 3 h culture by raising the temperature from 30 to 42 °C by immersion in a water bath. The cell density and GFP fluorescence intensity were measured 2 and 6 h after heat-shock induction.

To express foreign GFP, recombinant strains harbouring the pTH-GFPuv plasmid were grown at 30 °C in 50 ml of LB medium containing 50 μg/ml ampicillin using 250 ml flasks. These cultures were inoculated (1%, v/v) from 30 °C overnight cultures in the same medium. Heat shock was induced after 3 h culture by raising the temperature from 30 to 42 °C by immersion in a water bath. The cell density and GFP fluorescence intensity were measured 2 and 6 h after heat-shock induction.

Analytical assays

Cell growth was measured by monitoring attenuation (D) at 600 nm on a UV/visible spectrophotometer (UV-1601PC; Shimadzu, Kyoto, Japan). GFP fluorescence intensity was measured using a fluorescence spectrometer (RF-5301PC; Shimadzu) at an excitation wavelength of 395 nm and emission at 509 nm. Duplicate measurements were taken for each sample; average values of the duplicates were reported. In the case of stress responses, data reported include adjusted specific fluorescence intensity (SFI; raw fluorescence intensity divided by \(D_{600}\)), which is obtained by subtracting the specific fluorescence reading of *E. coli* containing the control pBR-GFPuv plasmid (gfpuv, gene without any promoters) from the fluorescence of the stressed-gfpuv-containing cells:

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\text{Adjusted SFI} = (\text{SFI}_{t} - \text{SFI}_{0})_{\text{stressed probe}} - (\text{SFI}_{t} - \text{SFI}_{0})_{\text{pBR-GFPuv}}
\]

where SFI\(_{t}\) and SFI\(_{0}\) are respectively the SFI at time t for measurement and at the start of the experiment. By using plasmid pBR-GFPuv as a negative fluorescence control, a determination of the intrinsic background fluorescence intensity of the cell itself was possible. In the case of foreign GFP expression, relative SFI values were calculated by dividing SFI under non-stressed conditions (30 °C) with SFI under heat-shock stressed conditions (42 °C).

SDS/gelatin PAGE (SDS/GPAGE) protease activity assay

Cellular protease activity was monitored with a modification of the gel electrophoresis method [13]. The frozen cell lysate supernatant samples were thawed at 37 °C. A mixture of 50 μl of Stop solution [0.123 M Tris/HCl, 2% (w/v) SDS, 20% (w/v) glycerol and 0.25 % Bromophenol Blue] and 50 μl of sample were vortexed and incubated at 37 °C for 20 min. After incubation, the samples were loaded into the wells of a slab polyacrylamide gel with SDS and gelatin co-polymerized into the matrix. After about 1 h at a constant voltage (200 V) the gels were washed in 2.5% Triton X-100 for 1 h at room temperature to remove SDS. The gels were then incubated at 37 °C for 24 h in an incubation solution (containing 100 μM glycine, 2 mM ATP and 2 mM MgCl\(_2\), pH 7.5) to regenerate protease activities. The gels were then stained for 1 h in 0.2% Amido Black staining solution and incubated in Amido Black destaining solution. SDS/GPAGE analyses were performed three times for convincing results. The stained gels were scanned and the digitized images were analysed by Gel-Pro Analyser software (Media Cybernetics, Silver Spring, MD, U.S.A.). Proteolytic activities were evident by clear bands appearing on the gel. The cleared zones were separated by relative molecular mass. The intensity of the cleared band was proportional to activity of the protease.

Results and discussion

Observation of cellular stress levels

Due to incorporation of the facile and non-invasive reporter GFP, we have successfully applied stress probes to monitor cellular stress responses in various stressed environments.
Comparison of cellular stresses in *Escherichia coli*

Heat shock was induced on 3 h-cultured cells by a temperature shift from 30 to 42 °C. The measurements of adjusted SFI were performed at 2 and 6 h after heat shock. Induction rates (adjusted SFI/h) of GFP expression were calculated 2 h after heat shock.

We investigated cellular stress levels in four *E. coli* strains, production hosts JM105 and BL21, and cloning hosts HB101 and TOP10, using three ‘stress probe’ plasmids (Figure 1). Cells were heat shocked for 2 h at 42 °C, and we observed rapid induction of stress-promoter-driven GFP expression (Figure 2A). Even though levels GFP expression declined slightly 2–6 h after heat shock in most cases, cellular stress levels generally exhibited similar patterns (Figure 2B). Expression of GFP from ClpB::GFPuv was induced at 2 h and rapidly down-regulated at 6 h in BL21, but not so dramatically in other strains. DnaK::GFPuv-driven GFP expression was induced at 2 h and rapidly down-regulated at 6 h in HB101, but not so dramatically in other strains. These observations of slightly different temporal stress patterns according to the stress probe could not be explained, but we should consider the error margin in measuring fluorescence intensity values using the stress probes. Therefore, since induction rates of GFP expression exhibited similar overall patterns with cellular stress levels, even though there were some slight differences depending upon the probe used, it was possible to compare cellular stress levels among *E. coli* strains.

The JM105 strain, which is widely used for foreign protein production, showed the highest cellular stress levels, regardless of the stress probe used. The levels of expression of the three probes in JM105 were pGFPuv-Sigma > pGFPuv-ClpB > pGFPuv-DnaK, which agrees with previously published findings using this strain [5]. BL21 was developed as a production *E. coli* strain through removal of proteases OmpT [14] and Lon [15], and our data show that it exhibited the lowest cellular stress levels. Interestingly, the data show that expression of protease subunit ClpB, stress signal factor σ32 and even chaperone DnaK were also low in BL21. These data suggest the BL21 strain might produce a much higher yield of foreign protein compared with other *E. coli* strains due to a lower stress response. In HB101 and TOP10 strains, which are widely used for DNA cloning work, cellular stress levels were lower than in JM105, but higher than in BL21 (Figure 2). In general, the amount of cellular stress observed in each strain can be ordered as such: JM105 > TOP10 > HB101 > BL21.

We performed SDS/GPAGE protease activity analyses to confirm the above data regarding stress levels in each strain (see Figure 3A). Even though almost all protease bands were not intense in all *E. coli* strains, it was possible to find a relatively clear protease band with a molecular mass of 26 kDa. According to a previous report, the 26 kDa protease had the strongest activity of all JM105 proteases, which number at least 24, including 12 cytoplasmic proteases [16]. Relative protease activity levels measured by scanning stained SDS/GPAGE gels are shown in Figure 3(B). SDS/GPAGE analysis showed that, with respect to the four strains, the order of cellular protease activity levels following heat shock was almost identical to that of cellular stress levels obtained using stress probes. These protease activity observations provide strong evidence that the stress probes can successfully indicate cellular stress levels in *E. coli*. In addition, they confirm the order of *E. coli* strains with respect to cellular stress as being JM105 > TOP10 > HB101 > BL21.
Observation of foreign protein expression abilities

We investigated cell growth and expression of a foreign protein in the four *E. coli* strains. GFP was also chosen as a model foreign protein due to its facile nature and because it can be easily quantitatively assayed [17,18]. GFP expression was determined by SFI. In a non-stress environment (culture maintained at 30 °C), BL21 showed the highest specific foreign protein yield, about 3.3-fold higher than that of JM105 (Figure 4). This may be expected since the BL21 strain showed the lowest cellular stress levels. BL21 showed higher cell density than JM105 and TOP10. The superior cell growth and foreign protein expression in BL21 (a derivative of *E. coli* B) compared with JM105 (a K12 derivative) observed in the current study are consistent with previous reports comparing BL21 and another K12 derivative, JM109 [19,20]. BL21 also shows lower accumulation of acetate, a known inhibitor of cell growth and foreign protein production [19]. For JM105, the expression levels were not satisfactory for a production host. The cell density of cloning strain HB101 was the highest of all strains, but GFP expression was the lowest, even though cellular stress levels were not high. This is consistent with the general belief that cloning strains are less able to express foreign proteins than production strains. However, our data revealed that even though cloning strain TOP10 showed a high cellular stress response, it expressed foreign proteins at greater levels than either HB101 or JM105. TOP10 grew at the slowest rate, which can be a critical negative aspect for large-scale foreign protein production.

We calculated relative cell growth and relative GFP expression levels in the four *E. coli* strains following heat-shock stress (42 °C; Figure 5). Relative values were calculated by dividing absolute values obtained under normal conditions (30 °C) with absolute values obtained under heat-shock conditions. Therefore, a value of 1 indicates that there are no effects, while values greater than 1 indicate positive effects and values less than 1 indicate negative effects. All strains showed similar growth under stress and normal conditions up to 4 h (except for JM105 which had slight increases in cell density; Figure 5A). After 8 h at 42 °C the cell densities of all strains decreased dramatically, in contrast to cells at 30 °C which kept growing. This growth arrest may be due to long exposure to heat shock and/or lower dissolved oxygen levels in the medium due to higher culture temperatures. The growth under heat-shock stress conditions showed the same order as that under non-stressed conditions: HB101 > BL21 > JM105 > TOP10 (results not shown). In JM105, which showed the highest cellular stress responses to heat shock (Figure 2), the specific GFP expression was reduced compared with that under normal conditions (Figure 5B). BL21, which showed the lowest cellular stress levels under heat shock and the highest specific GFP expression at 30 °C, increased specific GFP expression (about 3.6-fold increase at 8 h) during heat-shock stress, compared with growth at 30 °C. From these results, we conclude that heat-shock stress has a positive effect on foreign protein production in BL21. Similarly, but to a lesser extent, GFP expression in TOP10 and HB101 was also enhanced during heat shock. The reasons for enhanced expression under heat-shock conditions is not yet clear. One possible explanation is that higher culture temperatures...
result in a higher biosynthetic rate for foreign proteins, and that this enhanced biosynthesis rate exceeds the degradation rate under stress.

Conclusions
We compared cellular stress levels in E. coli strains using 'stress probe' plasmids that report on the activity of promoters for the stress genes transcription factor σ32, protease subunit ClpB and chaperone DnaK. JM105, a widely used production strain, showed the highest cellular stress levels. In contrast, BL21, another popular production host that lacks two predominant proteases OmpT and Lon, exhibited the lowest cellular stress levels. These stress probe results were confirmed by measuring protease activity using SDS/PAGE analysis. When we investigated expression of GFP as a model foreign protein for facile monitoring, JM105 showed low specific expression under non-stressed conditions, and even lower under heat-shock stress, compared with BL21. Interestingly, BL21 showed a great enhancement in specific expression yield and productivity under heat shock, even though cell density was decreased. We also observed that the cloning strain TOP10 showed much higher specific expression compared with JM105 and another cloning strain HB101, even though it had high cellular stress levels and the lowest cell density under both heat-shock stress and non-stress conditions.

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