Non-Invasive Environmental Detection using Heat Shock Gene – Green Fluorescent Protein Fusions

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Abstract
Three "stress probe" plasmids were constructed and characterized which utilize a green fluorescent protein (GFP) as a non-invasive reporter to elucidate *Escherichia coli* cellular stress responses in quiescent or "resting" cells. Facile detection of cellular stress levels was achieved by fusion of three heat shock stress protein promoter elements, those of the heat shock transcription factor σ^{32}, protease subunit ClpB, and chaperone DnaK, to the reporter gene gfp_{uv}. When perturbed by chemical or physical stress (such as heat shock, nutrient (amino acid) limitation, addition of IPTG, acetic acid, ethanol, phenol, antifoam, and salt (osmotic shock), the *E. coli* cells produced GFPuv which was easily detected from within the cells as emitted green fluorescence. A temporal and amplitudinal mapping of these responses was performed, demonstrating regions where quantitative delineation of cell stress was afforded.

Introduction
When exposed to chemical or physical stress, cells undergo many changes including alterations in the patterns of gene expression as well as protein stability. For example, when bacterial cells are exposed to high temperature, a set of heat shock proteins (hsps) are transcriptionally upregulated by transcription factor, σ^{32}. These hsps are evolutionarily well-conserved and many play an important role in the folding, assembly, degradation, and translocation of proteins, not only under stress conditions but also during normal cell growth. In addition to heat shock, a number of other stresses induce the synthesis of heat shock proteins in many organisms, such as viral infection, oxygen limitation, the presence of abnormal proteins, the overexpression of heterologous proteins, nutrient limitation (carbon source, amino acid source, etc.), and exposure to various chemicals including ethanol, phenol, hydrogen peroxide, and heavy metals.
In this report, we have utilized GFPuv as a reporter of cellular stress responses in *E. coli*. With GFPuv as a reporter, there is no requirement for ATP or other cofactors for fluorescence. Also, the metabolic requirements for generation of the GFP are minimal as GFP is relatively small (27 kDa). Stress was detected by transcriptional fusion of three heat shock stress protein promoter elements (those of heat shock transcription factor σ^{32}, protease subunit ClpB, and chaperone DnaK to the reporter gene gfp_{uv}. Because the native stress promoters were amplified from genomic DNA and incorporated into a plasmid such that they controlled the expression of GFPuv, we were able to quantify the cellular stress responses of *E. coli* by simply measuring GFP fluorescence intensity

Materials and Methods
Three heat shock promoters, specifically those of the heat shock transcription factor σ^{32}, protease subunit ClpB, and chaperone DnaK, were obtained by polymerase chain reaction (PCR) amplification from genomic DNA isolated from *E. coli* strain, K12. The K12 promoter/operator sequences for *rpoH* (for σ^{32}), *clpB*, and *dnaK* were obtained from the NIH GenBank. The -35 and -10 (TATA box) regions, transcription initiation site, ribosome binding site (RBS), and translation initiation site (codon: ATG) of the native proteins were used for regulation of GFPuv. That is, since the length between the RBS and translation initiation site is an important factor for translational efficiency of foreign protein expression, we used the translation initiation sites of the original stress

promoters. The gfp_{uv} gene was excised from the pGFPuv plasmid and was inserted into three pBR322 plasmid each containing a heat shock stress protein promoter. The recombinant plasmids pGFPuv-Sigma, pGFPuv-ClpB, and pGFPuv-DnaK contain heat shock stress protein promoters *rpoH*, *clpB*, and *dnaK*, respectively, and the gfp_{uv} gene.

Results and Discussion

1. Cellular Stress Response by Heat Shock

An increase in specific fluorescence intensity (SFI) was observed for the σ^{32}, ClpB, and DnaK plasmids in *E. coli* JM105 cells heat shocked by a temperature shift from 30°C to 42°C. Also, there was significant pre-stress fluorescence revealing the following pattern of intensity: pGFPuv-Sigma > pGFPuv-ClpB > pGFPuv-DnaK > pBR322. Since the RBS and promoter elements were included in the regulatory sequences of the fusions, increasing fluorescence intensity (pre and post stress) demonstrated increased transcription and/or translation of the σ^{32} transcription factor, protease subunit ClpB, and chaperone DnaK. In the case of the strain containing the pGFPuv-Sigma plasmid, the fluorescence levels increased within in the first 2 h after temperature shift. For the pGFPuv-ClpB and pGFPuv-DnaK plasmids, the fluorescence levels increased most after 4 h. The fluorescence level of *rpoH*::gfp_{uv} was always at least 50% greater than the *clpB*::gfp_{uv} and *dnaK*::gfp_{uv} under heat shock conditions. Also, because the raw fluorescence data were very similar to the specific fluorescence data, we concluded that there was virtually no interference due to the intrinsic fluorescence of the cells.

2. Dose-Response Curve for Ethanol Shock

Ethanol is an efficient inducer of heat shock proteins. We evaluated the ethanol dose-response profile for the pGFPuv-DnaK plasmid. At early times post ethanol addition, the fluorescence response was non-linear with ethanol at low concentrations (<4% (v/v)). The fluorescence was linear with ethanol at all times in the range of 2 to 4 % (v/v) and at later times, linearity was established over a wider range of ethanol (r^2 value of 0.989 at 8 h for 0 to 4% (v/v)). Since the slopes of fluorescence versus ethanol concentration below 4% (v/v) and of fluorescence versus time plots were both positive, the best sensitivity for backward discrimination of ethanol levels was afforded at 8 h, or at the end of our experiment. Interestingly, as the ethanol concentration was increased above 4% (v/v), an apparent toxicity threshold was reached wherein the DnaK-promoted GFPuv expression started to decrease at high ethanol concentrations.

3. Detection of Several Environmental Insults

The fluorescence levels of each strain insulted by several stresses were plotted. For all probes and for all but one stress, a positive response was observed. In the outlier case, IPTG was added at three concentrations to the ClpB strain. *E. coli* JM105 are *lacI*^{q} and there was no plasmid encoded *lac*-based promoter; in the absence of mRNA amplification (which might lead to stress), there was only a slight increase in fluorescence. This was consistent with a previous report demonstrating small increases in GroEL, DnaK, and GroES upon addition of IPTG in a *lac*$^-$ strain. With the exception of ethanol addition to the *rpoH*::gfp_{uv} strain, the response to each insult was highest for the *rpoH*::gfp_{uv} strain and lowest for the *dnaK*::gfp_{uv} strain. Also the increases observed for the *rpoH*::gfp_{uv} for serine hydroxamate, antifoam, and ethanol insults were not appreciably different that the increase in the unstressed control. Not shown in this figure are temporal results wherein σ^{32} always led the chaperone and protease subunit. Also, the addition of acetic acid (10 g liter^{-1}) quenched fluorescence for the σ^{32} and DnaK plasmids.