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Stress Responses of the *Escherichia coli groE* Promoter

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Abstract GroEL is well known as a molecular chaperone. In order to determine the dynamic stress response of the *Escherichia coli groE* promoter, a *groE-lacZ* operon fusion in the chromosome was constructed. Stress leading to σ^{32} synthesis induces transcription from *E. coli groE* promoter, since the promoter is σ^{32}-regulated. When the strain was stressed with ethanol, phenol, and sodium chloride, clear inductions of β-galactosidase were observed. Two types of simultaneous stresses of sodium chloride and phenol induced the enzme much more than either of the two alone, suggesting that stress was an additive. The combined stress resulted in the highest induction of the enzyme in this system. The *groE-lacZ* fusion strain developed in this study can conveniently be used to detect other harmful pollutants in the environment. Stress treatment of cells containing recombinant proteins, which need GroEL, by ethanol, phenol, or sodium chloride, might have a tendency to increase their biological activities.

Key words: *E. coli groE* promoter, *lacZ*, ethanol, phenol, sodium chloride

It is well known that the molecular chaperone, GroEL, is involved in assisting protein folding, assembly, secretion, and degradation [4, 13, 23]. GroEL is conserved in all kinds of bacteria, and its deletion mutant cannot be obtained, suggesting its essential role in the cell [2]. Most of the conducted study is concentrated on the interaction between GroEL and substrate proteins, and the study on *groE* promoter itself is not extensive. *E. coli groE* promoter is located upstream of the *groES-groEL* operon [6]. Stress, such as viral infection [15], nutrient limitation [8], and heavy metals [20] can induce stress proteins. Some stressful conditions can activate σ^{32}(RpoH) regulated promoters [1]. Since *groE* promoter is also σ^{32} regulated, much stress leading to σ^{32} synthesis can induce transcription from *groE* promoter. It was determined whether or not several types of stresses can induce transcription from *groE* promoter, and this was achieved by using *groE-lacZ* operon fusion in the chromosome. GroEL induction can be conveniently measured in the fusion strain indirectly by using the β-galactosidase assay instead of western blotting.

β-galactosidase downstream of *groE* promoter was induced by ethanol, phenol, and sodium chloride in this system in a stress-concentration dependent manner. Sodium chloride stress was effective in inducing the enzyme, and the combined stress of sodium chloride (5%) and phenol (2,000 ppm) was found to be most effective in the enzyme induction. The knowledge that ethanol, phenol, or sodium chloride can raise GroE induction can be exploited in preventing insolubility of overproduced recombinant proteins. In fact, these proteins definitely need more GroEL protein in their folding, and this was carried out by giving the cells with recombinant proteins some sort of stress.

The *E. coli* YH24 strain containing the *groE-lacZ* fusion can be developed further to detect harmful toxicants in the environment.

Materials and Methods

Construction of Fusion Plasmid and Bacterial Strain

Characterization of the *groE* promoter under stress conditions can be easily accomplished by using *lacZ* as a reporter. The *groE* promoter was amplified from *E. coli* DH5α strain using two primers. One was 5'AAGGAATT-CGATCAGAATTTTT3' containing an *Eco*RI site, and the other was 5'AAGGGATCCTTTGAGAAAGTCC3' containing a *Bam*HI site. The amplified product (139 bp) was cut with *Eco*RI and *Bam*HI, and then ligated into the *Eco*RI and *Bam*HI sites of plasmid pRS415 [16]. The ligation mixture was transformed into *E. coli* DH5α, and screening was completed for transformants. Plasmid pRS415 containing the *groE* promoter and ampicillin resistant gene was named pRSgro-lac (Fig. 1). In pRSgro-

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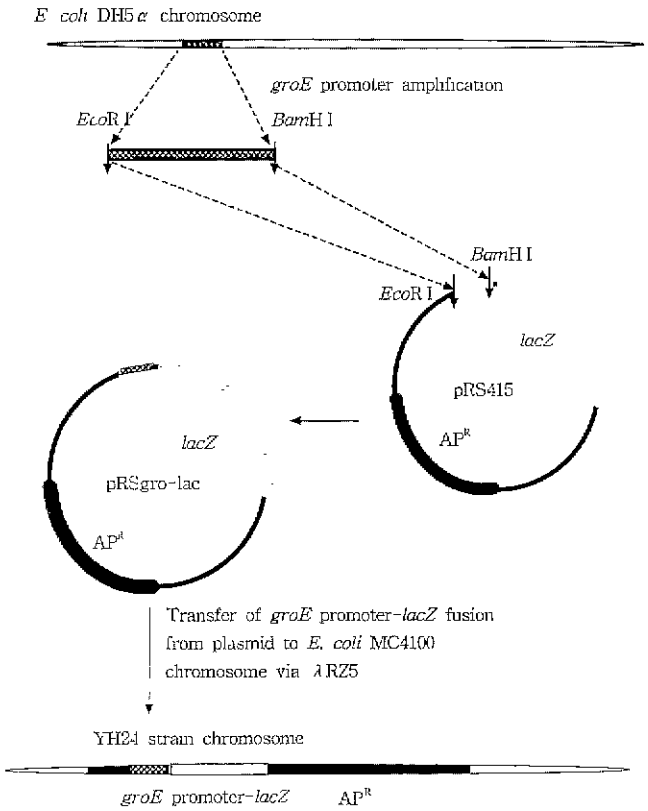

Fig. 1. Construction of a *groE-lacZ* fusion strain.
Details of construction are in the text. The strain containing the *groE* promoter-*lacZ* fusion is designated as *E. coli* YH24.

lac, the *lacZ* expression derivative of its native ribosome binding site is under the control of the *groE* promoter, which is a transcriptional fusion. When cells are stressed, β-galactosidase under the control of the *groE* promoter is synthesized. Copy number variations of plasmids in the cell, when stressed, can hinder the process of getting accurate measurements of the stress response. To overcome this problem, transcriptional fusion of *groE-lacZ* was transferred from the plasmid containing *lacZ* and the ampicillin resistant gene into the *E. coli* MC4100 chromosome, a *lac*-deletion strain [25], using homologous recombination of phage λRZ5 [14] and plasmid pRSgro-lac. *E. coli* MC4100 was lysogenized by the recombinant phage. The strain containing the *groE-lacZ* fusion was screened by ampicillin resistance and β-galactosidase expression (Fig. 1). The proper insertion of fusion was also confirmed by finding 293 bp of PCR amplified product just as it was predicted. One primer was 5'AAGGAATTCGATCAGAATTTTT3' whose sequence is in the *groE* promoter, and the second primer was 5'CGCCAGGGTTTTCCCAGTCACGA3' (*lacZ* universial primer). The recombinant strain was named *E. coli* YH24.

PCR and DNA Sequencing

PCR amplifications for obtaining two PCR products were carried out as described in 'Construction of a fusion plasmid and a bacterial strain' in the Perkin-Elmer thermal cycler (Norwalk, U.S.A.), with the following steps: an initial denaturation step at 95°C for 7 min; 30 cycles of denaturation (95°C for 1 min), annealing (45°C for 1 min), extension (72°C for 1 min), and a final extension step at 72°C for 5 min.

For sequencing the *groE* promoter portion on plasmid pRSgro-lac, the dideoxy termination method by ThermoSequenase (Amersham Life Science, Arlington Heights, U.S.A.) was used.

Measurement of Stress Induction

E. coli YH24 was cultured in LB (Difco, Detroit, U.S.A.), at 37°C for 14 h, was diluted 100-folds in a new LB, and then was cultured again to reach its exponential growth phase. At this phase, each culture was stressed by various concentrations of ethanol, phenol, sodium chloride, or sodium chloride plus phenol. β-galactosidase activity was determined by using ONPG (Sigma Chemical Co., St. Louis, U.S.A.) as a substrate [11] after stress exposure at appropriate intervals. The experimental results were obtained after at least three sample analyses.

Results and Discussion

E. coli groE Promoter-*lacZ* Fusion

Although GroEL can be induced by many stresses, quantitation of the protein is not convenient. Fusion of the *E. coli groE* promoter and *lacZ* allows for easy determination of stress responses of the *groE* promoter by the β-galactosidase assay. Stress promoter can also be fused into *luxCDABE* genes, and bioluminescene can be measured when cells are stressed [21]. However, the β-galactosidase assay detecting a yellow color used as a reporter is simple and sensitive enough to be used widely.

groE promoter sequences amplified by PCR from *E. coli* DH5α were inserted into the promoterless *lacZ* on the plasmid pRS415, which was named pRSgro-lac. The portion of *groE* promoter on the plasmid pRSgro-lac was sequenced and confirmed by the method of Sanger's dideoxy method (Fig. 2). Our Genbank accession number of the *groE* promoter sequence of *E. coli* DH5α is AF180938 Plasmid pRSgro-lac was transferred into an *E. coli lacZ* deletion strain using λ phage, which was named *E. coli* YH24 (Fig. 1). This strain can be conveniently used for determining stress responses of the *groE* promoter, since the *groE* promoter is fused into *lacZ* in the chromosome.

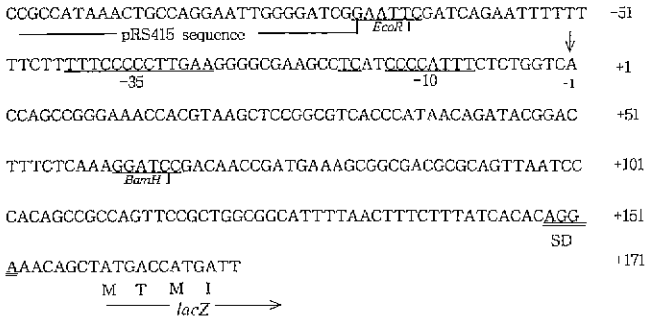

Fig. 2. Nucleotide sequence of *groE* promoter-*lacZ* fusion.
E. coli groE promoter was inserted into *Eco*RI and *Bam*HI sites of plasmid pRS415. The -35 and -10 regions of *groE* promoter and 'TC' in the spacer region are underlined. Transcription start site is marked as +1. SD is the Shine-Dalgarno sequence of *lacZ*, and MTMI are amino acid sequences deduced from the *lacZ* gene.

E. coli groE promoter of HB101 and Y1090 contains TTTCCCCCTTGAA in the -35 region, and CCCCATTT in the -10 region, which is one of the typical σ^{32} consensus sequences [7]. The *rpoH* gene product synthesized upon stress is σ^{32} which stimulates transcription initiation from *groE* promoter, one of the σ^{32}-controlled stress promoters.

The *groE* promoter sequence of DH5α was the same with the above-mentioned strains, and DH5α *groE* promoter has a 'TC' base between the -10 and -35 region, spacer region. Although Lindler [7] used dITP to find the 'TC' sequence in the spacer region of the *groE* promoter, two bases were found in this study even without using dITP (Fig. 2). There is a possibility that a secondary structure in the spacer region was destroyed when it was manipulated by high temperature from PCR denaturation steps in the sequencing process. In the *groE-lacZ* fusion, *E. coli groE* promoter was inserted into *Eco*RI and *Bam*HI sites of the plasmid, and it was followed by a Shine-Dalgarno sequence of *lacZ* (Fig. 2). Thus, transcription of the fusion gene was under the control of *E. coli groE* promoter, and translation was that of the *lacZ* gene.

Ethanol Stress

It is assumed that ethanol has an effect on the protein folding in the cytoplasm, or on the membrane integrity of cells [3]. Ethanol efficiently induces heat shock proteins [12]. The effect of ethanol on protein folding has been reported to be almost identical to that of σ^{32} on its folding [19]. It is known that σ^{32} regulates induction of about 20 stress proteins in *E. coli* [24]. Since the *groE* promoter is also σ^{32}-controlled, ethanol effect on *groE* induction was determined in this study. When *E. coli* YH24 containing the *groE-lacZ* fusion in the chromosome was stressed with 2% ethanol, the cell increased β-galactosidase expression conspicuously to the maximum level of about 1,360 U (Fig. 3, Table 1), compared to the control strain that was

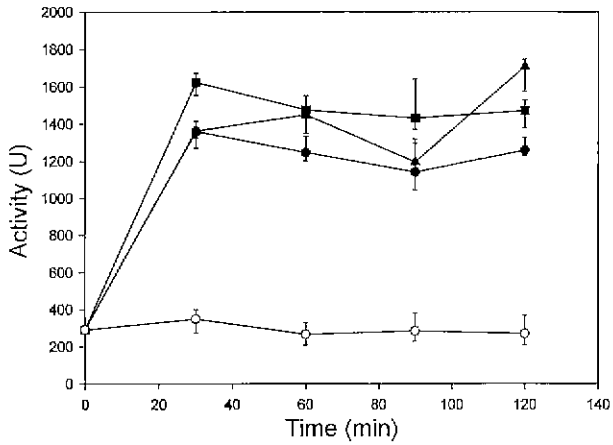

Fig. 3. Ethanol stress.
E coli YH24 which is lysogenic for the *groE-lacZ* fusion was stressed with 2% (●), 4% (■), and 6% (▲) ethanol at the exponential phase. β-galactosidase activities were measured at each intervals. *E coli* strain without *groE* promoter (○) was used as a control. Bars represent standard deviations.

Table 1. Effects of various types of stresses on *groE-lacZ* induction.

Stress	Concentration	Maximum activity (U)
Ethanol	2% (v/v)	1,360
	4% (v/v)	1,623
	6% (v/v)	1,708
Phenol	200 ppm	1,246
	1,000 ppm	1,455
	2,000 ppm	2,021
NaCl	3.5% (w/v)	1,685
	5% (w/v)	2,156
NaCl+Phenol	5% (w/v)+2,000 ppm	4,615
Strain without *groE* promoter		249

without a *groE* promoter (249 U). Furthermore, when the cell was stressed by 4% ethanol, β-galactosidase expression increased to the maximum level of 1,623 U (Fig. 3, Table 1), showing an ethanol-concenetration dependent response of the *groE* promoter. However, when the strain was stressed with 6% ethanol, the stress response was not concentration-dependent, suggesting that 6% ethanol is too toxic for these cells (Fig. 3). Generally, sublethal concentrations of stress do not significantly inhibit the cell's growth and, therefore, is appropriate in determining stress response.

It is shown in this study that ethanol treatment of cells can raise GroEL induction in these cells. It has earlier been reported that 3% ethanol treatment of cells indeed resulted

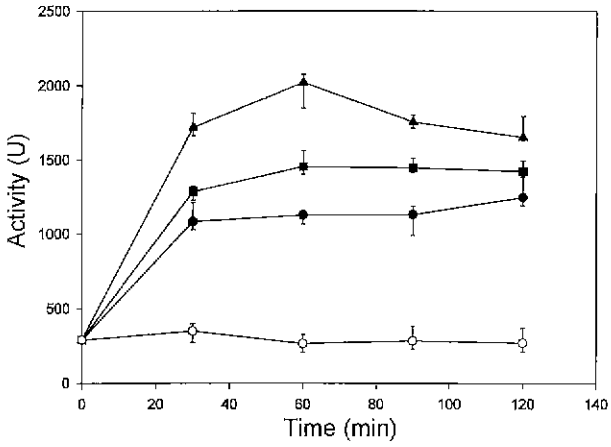

Fig. 4. Phenol stress.
E. coli YH24 lysogenic for the *groE-lacZ* fusion was stressed with 200 (●), 1,000 (■), and 2,000 ppm (▲) phenol at the exponential phase. *E. coli* strain without *groE* promoter (○) was used as a control. Bars represent standard deviations.

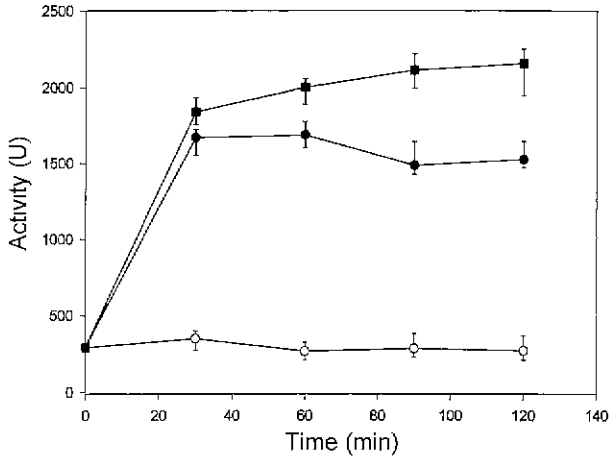

Fig. 5. Salt stress.
E. coli YH24 lysogenic for the *groE-lacZ* fusion was stressed with 3.5% (●) and 5% (■) sodium chloride at the exponential phase. *E. coli* strain without *groE* promoter (○) was used as a control. Bars represent standard deviations.

in a 2–3 fold increase in the activity of PreS2-S'-β-galactosidase, which was otherwise aggregation-prone, by increasing chromosome-encoded GroEL synthesis [19]. Ethanol treatment can also increase the yield of active lipoxygenase [17]. GroEL can prevent many recombinant proteins from becoming insoluble, thus, facilitating the production of active and soluble forms [4, 10]. Overproduction of σ^{32}, increase of the cell growth temperature, and addition of ethanol to the growth medium are all effective in increasing cellular levels of heat-shock proteins [18, 19].

Phenol Stress

Phenol is generally toxic to most organisms and some microorganisms are expected to produce stress proteins to protect themselves. There is a report that phenol strongly induced stress response from the *dnaK* promoter [21]. Therefore, the effect of phenol stress on *groE* promoter induction was determined in this study. When *E. coli* YH24 containing the *groE-lacZ* fusion in the chromosome was stressed by 200, 1,000, and 2,000 ppm of phenol, β-galactosidase expression increased to the maximum levels of 1,246, 1,455, and 2,021 U (Fig. 4, Table 1), respectively, as compared to 249 U in the control strain without *groE* promoter. Thus, stress response of the *groE* promoter depended on phenol concentrations. Since the outer membrane of bacteria is known to hinder transport of phenol into the cells [21], development of a strain which transports phenol easily into the cell of the *groE-lacZ* fusion will significantly contribute to a sensitive detection of phenol stress response.

Since it has been shown that the strain YH4 containing the *groE-lacZ* fusion is effective in detecting phenol, there is a possibility for the strain to be used for identifying other phenol-related toxicants such as 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), pentachlorophenol (PCP), and 2,4-dinitrophenol (2,4-DNP). Development of sensitive biosensors for detecting these toxic chemicals will definitely make a contribution in solving some or most of the environmental pollution problems easily and quickly.

Salt Stress

Hyperosmotic environments such as seawater or dry soils are extremely toxic to bacteria when they are deprived of water [5]. Raising the level of K^+ ions or trehalose inside of the cell is one of the major osmoadaptations [9]. However, osmoregulation by stress protein has not been extensively studied. Sodium chloride is a weak inducer of transcription from *E. coli grpE* or *uspA* promoters [22]. When *E. coli* YH24 carrying *groE-lacZ* in the chromosome was stressed with 3.5% and 5% sodium chloride, β-galactosidase was induced to the maximum levels of 1,686 and 2,156 U (Fig. 5, Table 1), respectively, in a salt-concentration dependent manner. In general, the values are higher than those obtained by ethanol stress, thus, indicating that the effect of salt stress on *E. coli groE* was relatively strong, compared to *grpE* or *uspA* induction. To the best of our knowledge, sodium chloride effect on *E. coli* GroE induction has not yet been reported.

Induction by Two Simultaneous Stresses

In the natural environment, a variety of pollutants are mixed, therefore, the effect of combined stresses of 5% sodium chloride and 2,000 ppm phenol on *E. coli* YH24 lysogenic for *groE-lacZ* fusion was investigated in this study. Combined stresses of sodium chloride and phenol

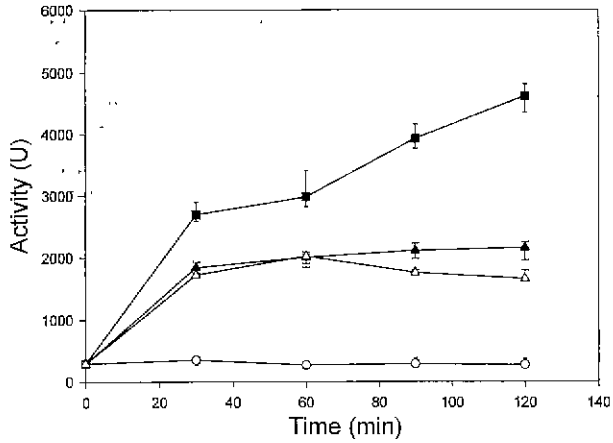

Fig. 6. Combined stresses of salt and phenol.
E coli YH24 lysogenic for the *groE-lacZ* fusion was stressed with 2,000 ppm phenol plus 5% sodium chloride (■), 5% sodium chloride (●), and 2,000 ppm phenol (△) at the exponential phase. *E. coli* strain without *groE* promoter (○) was used as a control. Bars represent standard deviations.

resulted in much more expression of β-galactosidase compared to either sodium chloride or phenol alone (Fig. 6), thus, indicating that the responses from the two types of stresses were additive. The two simultaneous stresses resulted in a maximum induction of β-galactosidase (4,615 U) (Fig. 6, Table 1), which was the highest value attained in this system. The second highest value was obtained by using 5% of sodium chloride (2,156 U) (Fig. 5, Table 1).

GroEL is inducible not only by heat shock but also by various kinds of stresses and it is properly called a stress protein in this sense. It is proven in this study that GroE synthesis in the cell can be enhanced by ethanol, phenol, and sodium chloride. These chemicals used for GroE induction inhibited the cell growth slightly. However, since they were used at sublethal concentrations, except for 6% ethanol, all GroE induction occurred in a concentration-dependent manner.

There are many recombinant proteins which require GroEL for their proper folding. Thus, addition of ethanol, phenol, or sodium chloride into the growth medium might increase their biological activities. Furthermore, the strain of *groE-lacZ* fusion can be used to detect other types of toxic stresses, since β-galactosidase expression under the control of the *groE* promoter can be conveniently measured.

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