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Rapid and Sensitive Pollutant Detection by Induction of Heat Shock Gene-Bioluminescence Gene Fusions

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Heat shock gene expression is induced by a variety of environmental stresses, including the presence of many chemicals. To address the utility of this response for pollutant detection, two *Escherichia coli* heat shock promoters, *dnaK* and *grpE*, were fused to the *lux* genes of *Vibrio fischeri*. Metals, solvents, crop protection chemicals, and other organic molecules rapidly induced light production from *E. coli* strains containing these plasmid-borne fusions. Introduction of an outer membrane mutation, *tolC*, enhanced detection of a hydrophobic molecule, pentachlorophenol. The maximal response to pentachlorophenol in the *tolC*$^+$ strain was at 38 ppm, while the maximal response in an otherwise isogenic *tolC* mutant was at 1.2 ppm. Stress responses were observed in both batch and chemostat cultures. It is suggested that biosensors constructed in this manner may have potential for environmental monitoring.

Two approaches to detection of environmental contaminants are currently practiced and required. Direct analytical methods allow determination of specific contaminants, and bioassays measure the harmful effects on living organisms. These biological toxicity tests commonly use fish or daphnia as the test organisms. In recent years, biochemical and microbial assays have gained importance because of rapid responses, low costs, and improved reproducibility. An example of such an approach for general toxicity detection is the measurement of metabolic death by loss of bioluminescence from the marine microorganism *Photobacterium phosphoreum* (7). In this report, an alternative approach to environmental monitoring based on detecting cellular adaptive responses is suggested.

Organisms frequently encounter environments that are not optimal for growth. In response to such stressful conditions, cells expend energy to maintain homeostasis via specific and general mechanisms, including alterations in gene expression. For example, the heat shock response, a process found in all organisms, is thought to allow cells to respond to protein unfolding (9, 16). When cells experience an abrupt increase in temperature, the synthesis of a number of evolutionarily conserved heat shock proteins is induced. These stress proteins include the molecular chaperones, Hsp60 and Hsp70, which have important cellular functions in protein folding and renaturation during both steady-state growth and heat shock (23). In *Escherichia coli*, transcriptional regulation by the positively acting RNA polymerase subunit, sigma-32, accounts for the heat shock induction of about 20 genes (24). These include *dnaK*, encoding Hsp70; *groEL*, encoding Hsp60; and *grpE*, encoding an essential protein known to interact with the *dnaK* and *groEL* gene products (2, 3, 15, 18). A hallmark of the sigma-32-mediated heat shock response is the rapid, but transient, increase in the relative rates of heat shock protein synthesis (17, 34).

In addition to heat, a number of other stress conditions induce synthesis of heat shock proteins in many organisms. Viral infection, a change from anaerobic to aerobic conditions, the presence of abnormal proteins, and exposure to various chemicals including ethanol, 2,4-dinitrophenol, sodium azide, hydrogen peroxide, heavy metals, and amino acid analogs are all known inducers (33). In *E. coli*, a subset of proteins induced by heat shock also is induced by various starvation conditions and by the presence of chemical pollutants (5, 12, 31). The induction of the heat shock response, typically observed at subinhibitory concentrations, is thought to be an important mechanism for survival in the presence of environmental insults. This common induction profile by diverse stresses suggests that monitoring the heat shock response may be a sensitive method for detecting environmental insults.

Facile detection of transcriptional activation is achieved by fusion of promoter elements to reporter genes. A particularly useful reporter system is made up of the structural genes, *luxCDABE*, of the bioluminescence operon derived from the marine bacterium *Vibrio fischeri* (20, 30). The bioluminescence reporter has advantageous properties such as rapid response, excellent sensitivity, large dynamic range, and noninvasive continuous measurements amenable to automated data collection with minimal manipulations. Requirements for light production from the proteins encoded by these reporter genes include expression in actively metabolizing cells at temperatures below 30°C (6, 11). In this report, we describe recombinant *E. coli* strains that allow detection of stress-producing environmental conditions by increased light production.

MATERIALS AND METHODS

Construction of plasmids and bacterial strains. Plasmid pUCD615 (27), containing the *V. fischeri luxC*, *luxD*, *luxA*, *luxB*, and *luxE* genes without a promoter, was the parent plasmid for two genetic constructions. Plasmid pRY002 contains a *dnaKp::luxCDABE* fusion. The *dnaK* promoter was obtained by PCR amplification (GeneAmp PCR Reagent Kit; Perkin-Elmer Cetus, Norwalk, Conn.) of the 182-bp segment

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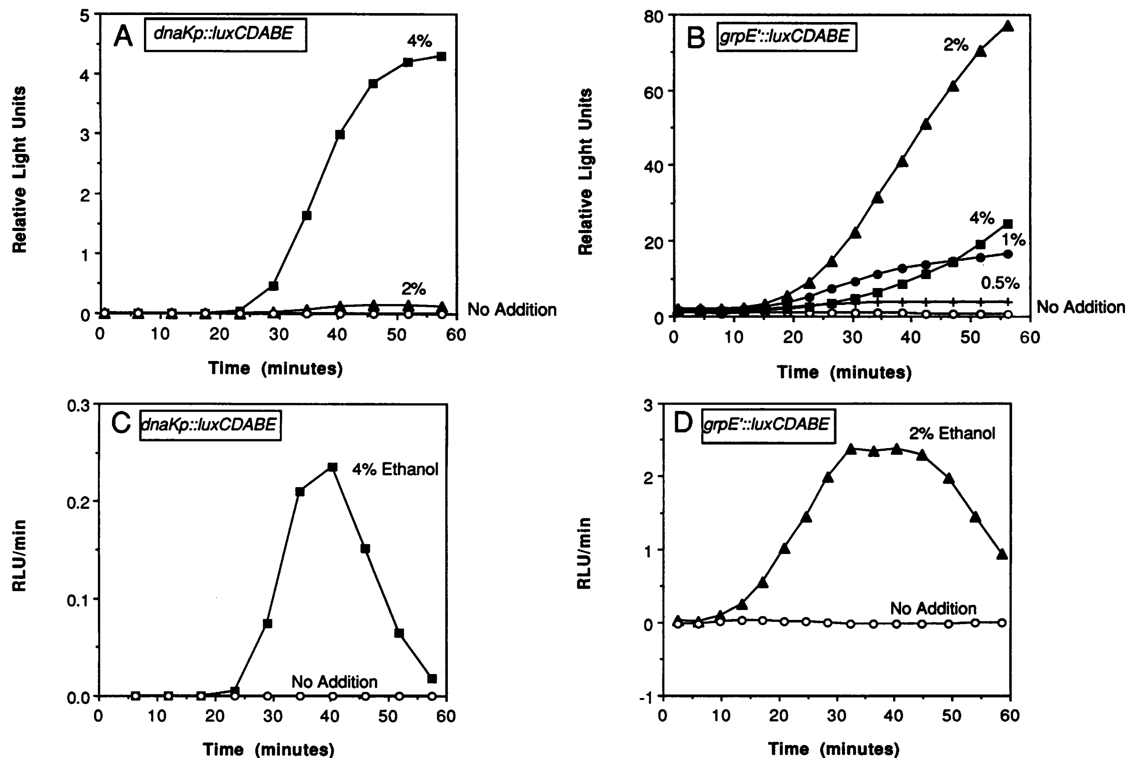

FIG. 1. Ethanol induction of the stress promoter-*luxCDABE* fusions. (A, B) Kinetics of light production. (C, D) Rate of increase of light production. *E. coli* WM1202 (*dnaKp*::*luxCDABE*) and TV1061 (*grpE'*::*luxCDABE*) were grown and tested at 25°C as described in Materials and Methods. Cells were added to ethanol to yield the indicated final concentrations at time zero. The light output values at time zero in the absence of ethanol were 0.002 RLU for strain WM1202 and 0.94 RLU for strain TV1061.

containing the *dnaK* promoter sequences (8) from lambda phage 9E4 of the Kohara collection (13), using primers 5'-GTTAGCGGATCCAAAAGCACAAAAAAT-3' and 5'-AGCAGTGAATTCCATCTAAACGTCTCCA-3', which allowed cloning (28) of the 192-bp *Bam*HI- and *Eco*RI-digested amplified product into the *Bam*HI and *Eco*RI sites of pUCD615. Plasmids pGrpELux.3 and pGrpELux.5 each contain a *grpE'*::*luxCDABE* fusion. These plasmids have a 628-bp *Hae*III to *Pvu*II restriction fragment containing 150 bp of the coding sequence and 480 bp of upstream sequences, including the promoter of the *E. coli grpE* gene (19), isolated from plasmid pGrpE4, a pUC18-derived plasmid containing the entire *E. coli grpE* gene. *Eco*RI linkers were ligated to the *Pvu*II end, and following digestion with *Eco*RI, the fragment was ligated into pUCD615 digested with *Sma*I and *Eco*RI (28). Plasmids pGrpELux.3 and pGrpELux.5 are independent isolates; the uninduced bioluminescence from cells containing plasmid pGrpELux.3 is less intense than that from cells containing plasmid pGrpELux.5 in several different *E. coli* hosts.

These plasmids were placed by $CaCl_2$ transformation (28) into two *E. coli* strains. *E. coli* RFM443 (*galK2 lac74 rpsL200*) has been described (21). *E. coli* DE112 is isogenic to strain RFM443 except for the mutation at the *tolC* locus. It was constructed by bacteriophage P1-mediated transduction (22), using a lysate grown on strain CS1562 containing the *tolC*::mini-Tn*10* mutation (4) as a donor and strain RFM443 (21) as a recipient. Resultant tetracycline-resistant recombinants were screened for hypersensitivity to the hydrophobic compound crystal violet (1). Strain WM1202 contains plasmid pRY002 in the RFM443 host, and strain WM1302 contains plasmid pRY002 in the DE112 host. Strains TV1060 and TV1061 contain plasmids pGrpELux.3 and pGrpELux.5, respectively, in the RFM443 host. Strain TV1076 contains plasmid pGrpELux.5 in the DE112 host.

Media, growth of cells, and chemicals. *E. coli* strains were grown to early log phase at 25°C in LB medium (GIBCO BRL) containing 50 μg of kanamycin monosulfate per ml following dilution from 25°C overnight cultures in the same medium. Culture turbidity was measured with a Klett-Summerson colorimeter. Cells were used immediately for stress induction tests following growth to Klett readings in the range of 15 to 30. Ethanol, methanol, phenol, and xylene were added directly to the LB-kanamycin medium. Stock solutions of pentachlorophenol (100 mg/ml in ethanol), 2,4-dichlorophenoxyacetic acid (100 mg/ml in ethanol), 2-nitrophenol (136 mg/ml in methanol), and 4-nitrophenol (112 mg/ml in methanol) were diluted such that the final concentration of solvent induced a negligible response. Copper sulfate was added either directly (for tests with strain WM1302) or from an aqueous stock solution (for strain TV1076). Continuous culture used a medium containing 20 g of glucose per liter, minerals, and trace elements (14) in a 14-liter light-proof bioreactor under the following conditions: dilution rate, 0.2 h^{-1}; pH 7.0; temperature, 30°C; dissolved oxygen concentration, >40% air saturation.

Measurement of bioluminescence. Light output was measured in a Dynatech ML3000 microtiter plate luminometer which was modified by the manufacturer to maintain a temperature below 30°C. Data were collected in the cycle mode at 25°C following addition of the cells (50 μl) to wells containing various concentrations of the compounds tested in the LB-kanamycin medium. The final volume in each well was 100 μl. Units of measurement were relative light units (RLU), which

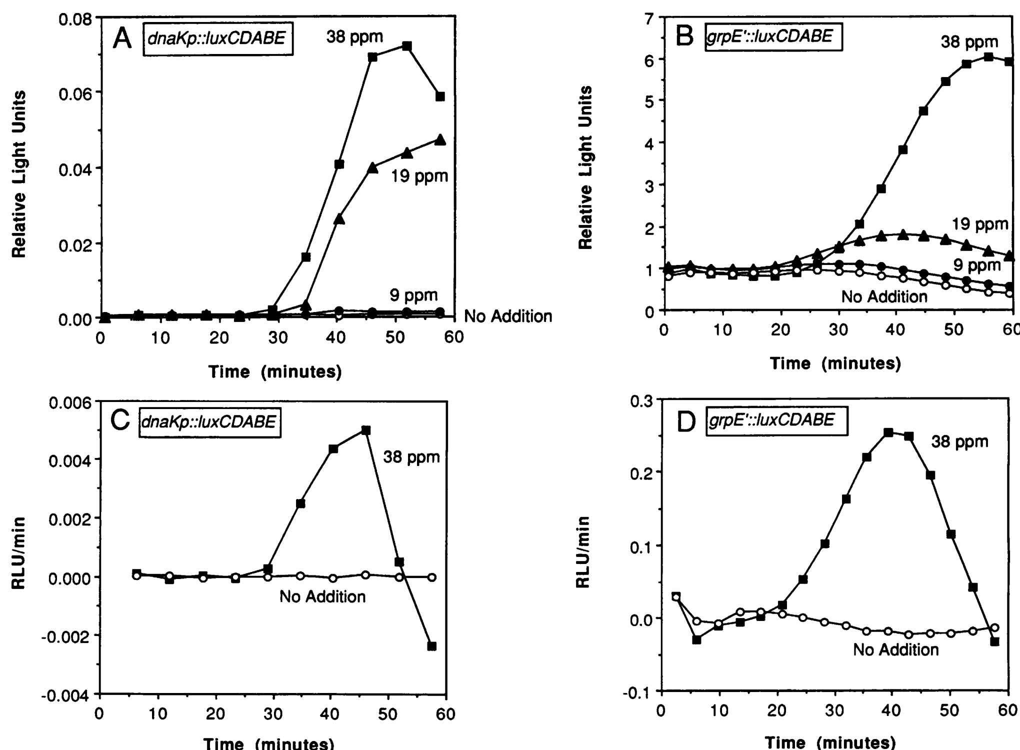

FIG. 2. Pentachlorophenol induction of stress promoter-*luxCDABE* fusions. (A, B) Kinetics of light production. (C, D) Rate of increase of light production. *E. coli* WM1202 (*dnaKp::luxCDABE*) and TV1061 (*grpE'::luxCDABE*) were grown and tested at 25°C as described in Materials and Methods. Cells were added to pentachlorophenol to yield the indicated final concentrations at time zero. The light output values at time zero in the absence of ethanol were 0.003 RLU for strain WM1202 and 0.80 RLU for strain TV1061.

were obtained by correction after comparison with the light reading from an internal light-emitting diode. Duplicate experiments for each concentration of chemical were conducted; average values of duplicates are reported. The rate of light increase (RLU per minute) was calculated by dividing the change in the RLU in a time interval by the minutes of that interval. Light output from cells grown in fermentors was quantitated in real time by a 20e luminometer (Turner Design, Sunnyvale, Calif.) equipped with a fiber-optic probe whose sensing end was plugged into one of the bioreactor ports (14). Units of measurement were arbitrary bioluminescence units.

RESULTS

Induction of bioluminescence. Operon fusions of two *E. coli* heat shock promoters, *dnaK* and *grpE*, to the *V. fischeri luxCDABE* genes were assembled in a multicopy plasmid (see Materials and Methods). *E. coli* transformants containing these fusions initially were characterized to confirm their response to ethanol, a chemical inducer of a strong heat shock response (24, 33). Increased bioluminescence was observed in the presence of ethanol from strains containing both the *dnaKp::luxCDABE* and *grpE'::luxCDABE* fusions (Fig. 1A and B). The intensity of the bioluminescence response varied with the concentration of ethanol added. At 60 min after addition of 4% ethanol, the strain containing the *dnaKp::luxCDABE* fusion was 1,900-fold brighter than the same strain in the absence of ethanol. The induction by ethanol was maximal at 2% for the strain containing the *grpE'::luxCDABE* fusion; at 60 min, this concentration of ethanol resulted in a 160-fold induction. The kinetics of induction were transient; in both cases, the rate of light production was maximal at about 40 min after addition of ethanol (Fig. 1C and D).

The Environmental Protection Agency priority pollutant pentachlorophenol also induced increased light production in strains containing both *dnaKp::luxCDABE* and *grpE'::lux CDABE* genetic fusions (Fig. 2A and B). The response to pentachlorophenol, while not as large in magnitude as the response to ethanol, was dependent on the dose of pentachlorophenol (Fig. 2A and B) and exhibited transient kinetics (Fig. 2C and D) in strains containing both stress promoter *lux* fusions.

Increased sensitivity with an outer membrane mutation. The effect of an outer membrane alteration on the responses to pentachlorophenol was examined. An isogenic pair of strains differing only by mutation of *tolC*, a gene known to affect permeability of the outer membrane to hydrophobic compounds, was constructed. Induction of the stress response from the *grpE'::luxCDABE* fusion was observed at substantially lower concentrations of pentachlorophenol in an *E. coli* strain containing a *tolC* mutation than in the *tolC*$^+$ control strain (Fig. 3). The maximal response in the *tolC*$^+$ strain was at 38 ppm of pentachlorophenol (the highest concentration tested). In the *tolC* mutant strain, the maximum response was at 1.2 ppm, and concentrations as low as 37 ppb resulted in detectable induction (response ratio = 1.4).

In contrast to induction of light production, a higher pentachlorophenol concentration resulted in inhibition of bioluminescence. The bimodal response of the *grpE'::luxCDABE* fusion in the *tolC* mutant'strain to pentachlorophenol can be seen in Fig. 3. Concentrations of up to 2.3 ppm resulted in induction of light production (response ratio, >1); at higher

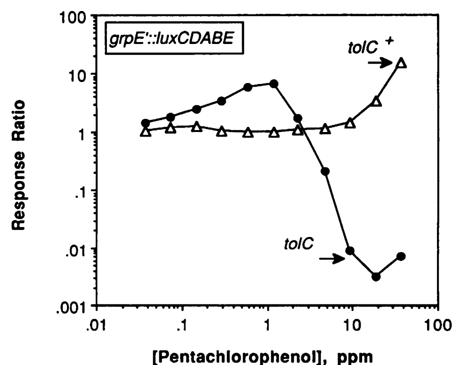

FIG. 3. Comparison of *tolC* and *tolC*$^+$ strains. Response ratios were calculated as the light measured at 60 min in the sample containing pentachlorophenol divided by the light measured at the same time with no addition. Shown are the effects of various concentrations of pentachlorophenol on the *E. coli* strains containing the *grpE'*::*luxCDABE* gene fusion, i.e., *tolC*$^+$ strain TV1061 and *tolC* strain TV1076.

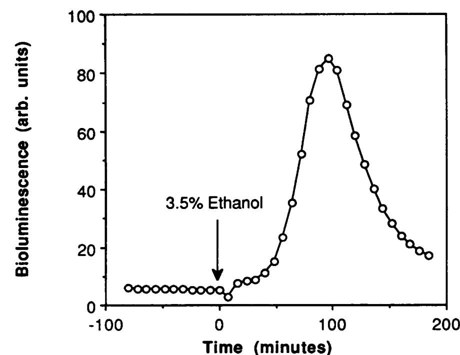

FIG. 4. Ethanol-induced stress response of cells grown in a continuous, glucose-limited chemostat. *E. coli* TV1060 containing a *grpE'*::*luxCDABE* fusion was grown at 30°C as described in Materials and Methods. The stress response was induced by a pulse addition of 3.5% ethanol to the chemostat at time zero.

concentrations, light production was inhibited (response ratio, <1).

Broad response profile. The responses of these strains, containing the *tolC* mutation and the stress promoter bioluminescence gene fusions, to other pollutant molecules are summarized in Table 1. Increased light production (response ratio, >1) was observed after addition of copper sulfate, 2,4-dichlorophenoxyacetic acid, methanol, 2-nitrophenol, 4-nitrophenol, and phenol for strains containing both the *dnaKp*::*luxCDABE* and the *grpE'*::*luxCDABE* fusions. Also, the strain containing the *grpE'*::*luxCDABE* fusion had increased light production in the presence of xylene. In general, for concentrations less than those giving the maximum response, a dose dependency of the response ratio versus concentration was present. In strains containing the *grpE* fusion, inhibition of light output (response ratio, <1) at higher concentrations was observed for most of the chemicals tested. Strains containing the *dnaKp*::*luxCDABE* fusion had very low basal levels of bioluminescence; therefore, loss of bioluminescence could not be determined accurately.

Stress response of cells grown in continuous culture. Use of the gene fusion-based biosensors requires a source of actively metabolizing cells. The above results demonstrate that batch-cultivated cells are capable of responding to stress. However, a constant source of growing cells, as could be obtained from continuous culture, would be more useful for many potential applications. Of the various continuous cultivation regimens, substrate-limited chemostats are convenient. However, it was uncertain whether this approach would be possible for several reasons. Starvation for carbon is known to induce a subset of the heat shock proteins (12). Cells growing in a chemostat already might be induced for heat shock proteins such that further induction would be difficult to observe. Moreover, growth under carbon limitation might result in decreased pools of the reduced cofactors necessary for light production. To test the feasibility of detecting stress responses with cells from continuous culture, *grpE'*::*luxCDABE*-containing cells were grown in a glucose-limited chemostat (see Materials and Methods). Despite the glucose limitation, these cells remained capable of responding to a model environmental stress, the addition of ethanol (Fig. 4).

DISCUSSION

Biosensor strains containing heat shock gene-bioluminescence gene fusions are capable of reporting environmental stress by induction of light production. The response of cells containing both the *dnaKp*::*luxCDABE* and the *grpE'*::*lux CDABE* fusions to a known stressing agent, ethanol, demonstrates the usefulness of this system for detection of stress to bacterial cells. The response was typical of that expected for a heat shock response by two criteria: a lag time prior to induction (approximately 20 min) was observed (Fig. 1A and B), as would be anticipated from the requirements for transcription and translation at 25°C; and the rate of the response was transient (Fig. 1C and D), as is typical of heat shock responses. The response of these biosensors to a pollutant, pentachlorophenol, was also consistent with a typical heat shock response. Both a lag time prior to induction (Fig. 2A and B) and transient induction kinetics (Fig. 2C and D) were observed. The magnitude of the response to ethanol was substantially greater than that to pentachlorophenol; however, this result was not unexpected because ethanol is the most potent known inducer of the bacterial heat shock response other than thermal shock (24).

The outer membrane of enteric bacteria, such as *E. coli*, effectively excludes hydrophobic compounds from the cell (25). Since induction of the heat shock regulon most probably requires intracellular localization of pollutant molecules, alterations allowing pollutant access were expected to extend the range of detected chemicals and reduce the detection limit. Other bioassays use bacterial outer membrane mutations, notably, *rfa* and *gal* mutations in the *Salmonella* mutagenicity assay systems (1), for this purpose. Mutations in the *E. coli tolC* locus, encoding a minor outer membrane protein, have also been used to increase permeability of hydrophobic compounds and have advantages such as genetic stability (10, 26, 29). The observation that the amount of pentachlorophenol that induces a significant stress response is much lower in strains containing a *tolC* mutation (Fig. 3) demonstrates its usefulness in this system. However, the magnitude of the increased bioluminescence is not as great in the *tolC* mutant as in the isogenic parent. This result was not completely unexpected, because the *tolC* mutation is known to have a number of pleiotropic effects (32). Nevertheless, the presence of the *tolC* mutation allows these strains containing heat shock gene-bioluminescence gene fusions to be sensitive detectors of low concentrations of hydrophobic chemicals.

TABLE 1. Effects of various pollutants on light production by *E. coli* strains containing heat shock gene-bioluminescence gene fusions[a]

Chemical	*dnaKp::luxCDABE*		*grpE'::luxCDABE*	
	ppm	Response ratio	ppm	Response ratio
Copper sulfate	93	0.9	250	1.3
	278	0.8	500	1.8
	833	**8.4**	**1,000**	**5.5**
	2,500	0.6	2,000	1.2
			4,000	0.0
2,4-Dichlorophenoxyacetic acid	12.5	1.1	1.6	1.6
	25	1.5	5	2.0
	50	17.8	15	3.6
	100	**108**	44	6.3
	200	4.5	**133**	**9.3**
	400	0.4	400	1.8
Methanol	2,500	1.2	2,000	1.3
	5,000	1.5	4,000	1.8
	10,000	1.3	8,000	3.2
	20,000	1.2	16,000	8.2
	40,000	**3.9**	**32,000**	**17.3**
			64,000	0.4
			128,000	0.0
2-Nitrophenol	42	1.0	13	1.4
	85	0.9	38	2.1
	170	1.2	**113**	**2.6**
	340	**6.2**	**340**	**2.6**
4-Nitrophenol	7.8	0.8	4.2	1.4
	16	1.0	13	1.9
	31	0.9	**37**	**3.1**
	62	**1.7**	111	0.09
			333	0.07
			1,000	0.0
Phenol	17	1.3	17	2.7
	50	2.2	50	4.7
	150	2.0	150	6.7
	460	9.1	**460**	**11.1**
	1,390	**68.2**	1,390	3.5
	4,170	0.6	4,170	0.0
Xylene	310	0.7	310	1.4
	620	0.6	620	1.7
	1,250	0.6	**1,250**	**1.8**
	2,500	0.5	2,500	1.4
	5,000	0.3	5,000	0.2
	10,000	0.3	10,000	0

[a] *E. coli* WM1302 (*dnaKp::luxCDABE tolC*) and TV1076 (*grpE'::luxCDABE tolC*) were grown and assayed as described in Materials and Methods. The response ratio was calculated by dividing the light output at 60 min in the presence of the chemical by the light output from an otherwise identical sample without addition. Thus, ratios of <1 represent inhibition of light production and ratios of >1 represent induction of light production. The conditions resulting in the maximum response ratios are shown in boldface.

The *grpE* and *dnaK* fusions were expected to behave similarly because both *dnaK* and *grpE* are controlled by sigma-32, and both encode proteins involved in protein folding (15). In general, the same compounds induced responses from both fusions. One difference between the two strains was that the uninduced light production from the *dnaK* fusion was much lower than that from the *grpE* fusion. This had the practical effect that small positive response ratios were more likely to be significant for the *grpE* fusion, and inhibition of light production was more readily observed with strains containing the *grpE* fusion. Other differences between the two fusions were that the maximum response ratio was typically greater for the *dnaK* fusion than for the *grpE* fusion, but the range of concentrations eliciting a response was typically broader for the *grpE* fusion.

The robustness of the bioluminescence reporter system under the various stress conditions was an initial concern. In fact, light production was inhibited by the presence of many chemicals (Fig. 3 and Table 1). This inhibition is presumably due to prevention of cellular metabolism responsible for energy and reducing power generation required for the bioluminescence reactions (20), inactivation of the enzymes involved in bioluminescence, or both. In accordance with this, the kinetics of light inhibition differed from that of light induction. While induction exhibited a lag time, the loss of

light production typically began immediately and was complete within the first 10 min (data not shown). Nevertheless, sublethal concentrations of many chemicals did induce bioluminescence, thereby demonstrating that the bioluminescence reporter system has sufficient robustness to allow detection of stress.

At concentrations of a given chemical less than that which induces the maximal response, the degree of induction was typically decreased with decreasing inducer doses. The dose-response relationship in this range was not linear but rather tended to increase dramatically as the maximal inducing dose was approached. The simplest interpretation of this response is that the stimulus level, presumably the presence of non-native proteins, was dramatically increased as the dose of the inducing agent neared toxic levels. In accordance with this hypothesis, concentrations somewhat greater than that which gave maximal induction often had bioluminescence increases less than the maximum (Fig. 3, for example), possibly reflecting a balance between induction of the stress response and toxic effects of the inducer. These biosensors have the advantage of two means of toxicant detection in environmental samples: induction of the stress response at lower concentrations and inhibition of light production at higher concentrations. Since the potential for both inducing and toxic responses exists, it is necessary to determine which dominates. This could be done by testing several dilutions of a given sample or including a control bioluminescent strain that does not respond to stress. Such a system has the potential to be generally applicable to the detection of environmental conditions which produce stress or are toxic to cellular metabolism.

Approaches such as this might be useful for several environments and processes. Thus, it may be desirable to customize the biosensors for a given environment through several approaches. We have shown that this system can be geared to various concentration ranges by mutational modification of the outer membrane. Furthermore, there is a possibility of customizing the pollutant detection profile by promoter choice from different global regulatory systems. Other important modifications may include introducing these or similar genetic constructs into other bacterial strains more representative of an environment to be monitored. Furthermore, use of bioluminescence as a reporter and biosensors grown in continuous culture allows the potential for real-time, continuous monitoring which could be applicable to computer control.

As environmental quality concerns mount, the need for rapid and facile assay methods capable of detecting environmental insults will increase. Induction of stress promoters that respond to a wide variety of hostile environments promises to be useful as a first indicator of the presence of such environmental insults. Detection of these responses will not require that all possible contaminants in a sample be known or that all possible interactions be predictable, but rather could give an early warning of adverse effects. Further tests could then be conducted to determine the presence of specific contaminants. Thus, these bioluminescence biosensors may be complementary to others currently being developed for detection of specific chemicals. Future research interests include characterization of such systems for the degree to which they detect the summation of the total toxic load and hence can serve as a trigger for subsequent diagnostic and remedial actions.

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