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Induction of Kanamycin Resistance Gene of Plasmid pUCD615 by Benzoic Acid and Phenols

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Abstract A *kan'::luxCDABE* fusion strain that was both highly bioluminescent and responsive to benzoic acid was constructed by transforming *E. coli* strain W3110 with the plasmid pUCDK, which was constructed by digesting and removing the 7-kb *Kpn*I fragment from the promoterless *luxCDABE* plasmid pUCD615. Experiments using buffered media showed that this induction was dependent on the pH of the media, which influences the degree of benzoic acid protonation, and the expression levels seen are likely due to acidification of the cytoplasm by uncoupling of benzoic acid. Consequently, the sensitivity of this strain for benzoic acid was increased by nearly 20-fold when the pH was shifted from 8.0 to 6.5. Benzoic acid derivatives and several phenolics also resulted in significantly increased bioluminescent signals. Although these compounds are known to damage membranes and induce the heat-shock response within *E. coli*, bacterial strains harboring mutations in the *fadR* and *rpoH* genes, which are responsible for fatty acid biosynthesis during membrane stress and induction of the heat-shock response, respectively, showed that these mutations had no effect on the responses observed.

Key words: Kanamycin, benzoic acid, uncoupling, bioluminescence, *aac(6')*-Ib

Benzoic acid as a weak acid is generally used in the preservation of food and beverages, since it is in its nondissociated form at acidic pHs that can freely pass across biological membranes and dissociate neutral pH of the cytoplasm, thereby disrupting the pH gradient [4]. This disruption results in the loss of oxidative phosphorylation, which in turn inhibits the growth of microorganisms. Many studies have focused on the effects of benzoic acid on fermentative yeast [14, 28–30]. In some of the studies, it was found that the rate of fermentation was stimulated as the benzoic acid concentration increased; however, at even higher concentrations, it was inhibited along with a loss of intracellular ATP and the accumulation of benzoic acid in the cells. Further investigation found that glycolysis was inhibited, accompanied with a lack of ATP utilization [30], thereby leading to bottlenecks in the pyruvate kinase and glyceraldehyde dehydrogenase-phosphoglycerate kinase steps [29].

More recent studies investigated the effects of benzoic acid on the inhibition of pathogenic *E. coli* strains in foodstuffs [7, 13, 18], and several studies found that numerous proteins of nonpathogenic *E. coli* strains are induced after the cells are exposed to benzoic acid [15, 31]. Many of these proteins are involved in amino acid synthesis and the heat-shock response. Furthermore, the *aph*(3')-IIb gene of *Pseudomonas aeruginosa*, which confers resistance to several aminoglycoside antibiotics, including kanamycin and neomycin, has been shown to be induced by the addition of 4-hydroxyphenylacetic acid [32].

Whereas the genes involved in kanamycin biosynthesis are known and have been characterized [33], little is known about the mechanisms controlling the expression of the kanamycin resistance gene(s) in *E. coli*. One study shows that mutations in the *hns* gene (referred to as *bglY* in the publication), which encodes for the H-NS protein of *E. coli* [19], led to an enhanced expression of the kanamycin resistance gene in plasmid pGR71 [3, 17], showing about 4-fold increase in the kanamycin resistance when this gene was mutated. On the other hand, the results with different kanamycin resistance genes, *i.e.*, the genes encoding for nucleotidyltransferase, phosphotransferase, or acetyltransferase, showed that this induction was not always observed, indicating that the origin of the gene may play a role in its control by the H-NS protein.

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Plasmid pUCD615, carrying a promoterless *Vibrio fischeri luxCDABE* operon [24] and conferring resistance to ampicillin and kanamycin, has been used in numerous studies to characterize promoter activities using the production of bioluminescence. Indeed, this plasmid has been used to construct numerous recombinant *E. coli* strains that can detect and respond to DNA damage [11, 21, 27], oxidative damage [2, 16, 20, 22], and heat-shock stress [20, 25, 26]. Furthermore, because of its broad host range application, it has also been used in the construction of other bacterial strains, including the development of several *Pseudomonas* strains that respond to naphthalene and salicylic acid [5, 10], phosphate availability [9], and BTEX chemicals [1].

In this study, the kanamycin gene of plasmid pUCD615 was fused with the *lux* genes present within the same plasmid. The *E. coli* strain carrying this plasmid was found to be highly bioluminescent and responsive to benzoic acid and its derivatives. It was found that the expression of the kanamycin resistance gene in plasmid pUCD615 was induced when *E. coli* was exposed to benzoic acid. Tests with buffered media proved that this induction was a direct consequence of uncoupling of benzoic acid, leading to a reduced cytosolic pH.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The *E. coli* hosts used in this study were strains W3110 [λ-, In(*rrnD-rrnE*), *rph-1*] [12], WL2 [*fadR201*, *tyrT58*, *adhEp-81*, *adhE2*, *mel-1*], ID18 [*hns-206*, *cysB9999*, *purC50*, *tyrA2*, *rpsL125* (*strR*), *thi*-1] [8], SC122 [*lacZ53*(Am), *phoA5*(Am), λ-, *tyrT47*(OS), *tyrT91*(ts, OS), *trp-48*(Am), *relA1*, *rpsL150* (*strR*), *malT66*(Am, λ^R), *spoT1*], and its *rpoH* isogenic strain K165 [*lacZ53*(Am), *phoA5*(Am), λ-, *tyrT47*(OS), *tyrT91*(ts, OS), *trp-48*(Am), *relA1*, *rpsL150* (*strR*), *malT66*(Am, λ^R), *rpoH61*(Am), *spoT1*] [23]. All five strains were kindly provided by the *E. coli* Genetic Stock Center (CGSC). Each was transformed with plasmid pUCDK.

Strains were initially grown overnight in 4 ml of sterile LB media (pH=7) in a 15-ml test tube at 30°C in a rotary shaker set at 250 rpm. The medium was supplemented with 100 μg/ml ampicillin for plasmid maintenance. For the 96-well experiments, 300 μl from each of the cultures was transferred to 50 ml of sterile LB with ampicillin in a 250-ml flask and grown under the same conditions as the seed. As A_{600} (Lambda 12, Perkin-Elmer, U.S.A.) reached 0.08, 100 μl of the culture was transferred to the wells of an opaque 96-well plate (Microplate 1, Dynex Inc., U.S.A.) into which the test chemicals had already been serially diluted. These chemicals were first diluted in LB and then serially diluted within the wells of the plate, with the final volume within each well (before addition of the culture) being 100 μl. The plate luminometer (Microtitre Plate Reader, MLX, U.S.A.) was set at 30°C, and the plate shaker was set to high and a one-second duration before each reading, which was done automatically every 10 min. The results were analyzed using Microsoft Excel™ and presented as either the bioluminescence or the relative bioluminescence, which is defined as the ratio of the sample's BL to that of the control at the same time point.

To study the effects of media pH on the responses of W3110/pUCDK, either MOPS or TAPS was added to final 100 mM concentration prior to subculturing of the bacterial culture. The pH of the unbuffered media was 7.2, whereas the pH of the media containing MOPS and TAPS was 6.5 and 8.2, respectively. Exposure to benzoic acid was performed as described above.

Plasmid Construction

All DNA modifying enzymes used in this study were purchased from New England Biolabs (NEB), U.S.A. Plasmid pUCD615 was digested with *Kpn*I, and a 10.5-kb fragment was religated, removing a 7-kb fragment that included the ori Sa and COS regions. This ligated plasmid,

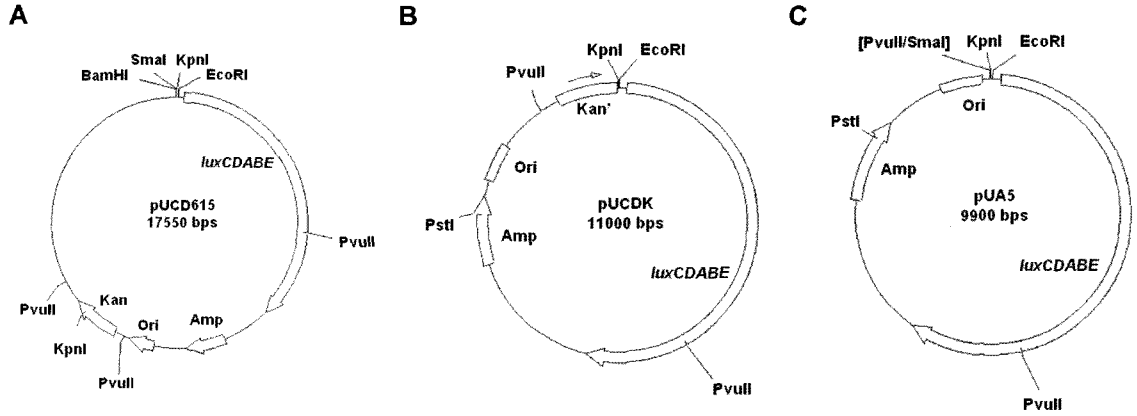

Fig. 1. Plasmids used in this study.

pUCDK (Fig. 1), was then transformed into *E. coli* strain W3110, and a single colony was selected after growth overnight on LB agar supplemented with ampicillin, and named W3110/pUCDK. To demonstrate that the response was due to the kanamycin gene, and not the ampicillin gene or the origin of replication, plasmid pUA5 (Fig. 1C) was constructed by digesting pUCD615 with PstI and SmaI and ligating an 8.3-kb fragment with the 1.6-kb fragment from a *Pst*I and *Pvu*II digest of the same plasmid, thereby removing the entire kanamycin gene and placing the expression of the *luxCDABE* genes upstream of both the ampicillin gene and the origin of replication.

mRNA Preparation and Real-Time PCR Analysis

After two 50-ml cultures of W3110/pUCDK were grown to an A_{600} of 0.08, benzoic acid was added to one of the flasks to a final concentration of 1.9 mM. Samples from each culture, *i.e.*, the unexposed control and the exposed sample, were collected, and the total mRNA was purified using the RNAprotect® and RNAeasy Mini® kits (Qiagen, U.S.A.), according to the manufacturer's protocol. The mRNA concentration of each sample was determined and diluted to attain the same concentration. Three aliquots from each mRNA sample were then reverse transcribed using the Taqman Reverse Transcription Reagents (Applied Biosystems, U.S.A.) and random hexamer primers. These replicate samples were used for Real-Time PCR (RT PCR) analysis.

RT-PCR was performed using the Taqman method where specific primers were ordered for both the *luxC* and *luxA* genes. The primers and probe for *luxA* were LUXA-LA2F 5'-AGGGCTACCAATGGTTCTTAGTTG-3', LUXA-LA2R 5'-TCATGACCATATTCTGTCGCAATTTCA-3', and LUXA-LA2M1 FAM-AAGCACA GATGGAACTCTAT-NFQ, respectively, and for *luxC* they were LUXC-LC2F 5'-TGC AAACGCTTTAGTTTCCAGTTTT-3', LUXC-LC2R 5'-TGCGGCCAATACATAAC AGACAT-3', and LUXC-LC2M1 FAM-ACCATCCAATAACCAAATCA-NFQ. The amplification reaction was carried out as suggested by Applied Biosystems (U.S.A.), using an ABI PRISM® 7000 Sequence Detection System from the same company. The relative mRNA concentration was determined using the relationship $RC=2^{(Ct(sample)-Ct(control))}$, where RC is the relative concentration and Ct(sample) and Ct(control) are the number of cycles needed for the sample's and control's fluorescence to exceed a given value, respectively.

Chemicals

All the chemicals used in this study are listed in Table 2. All, except phenol, were purchased from Sigma-Aldrich Chemical Company. Phenol was purchased from Yakuri Pure Chemical Co., Japan. Absolute ethanol used in the stock solution, when needed, was purchased from Merck Co., U.S.A. The alcohol concentration in the samples was less than 0.2%, a level that did not show any significant response from any of the strains (data not shown).

RESULTS AND DISCUSSION

Response of W3110/pUCDK to Benzoic Acid and Salicylic Acid

The pUCD615 plasmid was initially digested with KpnI to construct a different strain that was to be responsive to benzoic acid. However, the 11-kb self-ligated fragment, *i.e.*, without the 7-kb *Kpn*I fragment, when transformed into *E. coli* strain W3110, was found to be both highly bioluminescent and strongly dose-dependent inducible when exposed to benzoic acid and salicylic acid (Fig. 2). The minimum detectable concentrations (MDC) for both benzoic acid and salicylic acid, *i.e.*, concentrations that led to 2-fold induction in the BL over the control, at pH 7.2 were

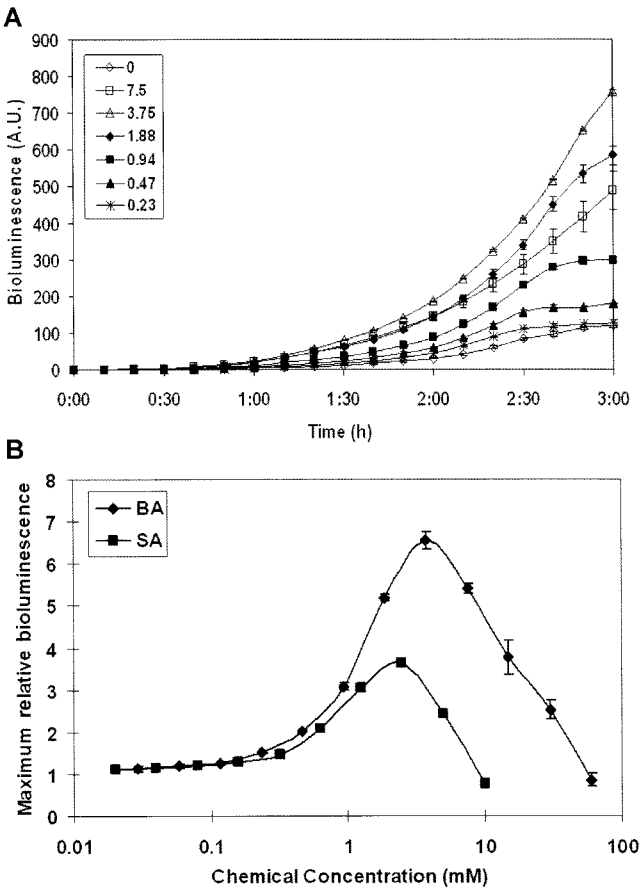

Fig. 2. A. Time-dependent bioluminescent responses from strain W3110/pUCDK when exposed to different concentrations (mM) of benzoic acid. **B.** Maximum relative bioluminescences (RBL) observed from strain W3110/pUCDK when exposed to benzoic acid (BA) and salicylic acid (SA).
A.U., Arbitrary Units. All samples were analyzed in triplicate for error analysis and the standard deviations are shown as error bars.

Table 1. Results from Real-Time PCR with the *luxA* and *luxC* primers. Values are relative to those of the unexposed control and were determined using the C_T values as described in Materials and Methods. Values in parentheses are standard deviations from three samples.

Time (min)	Gene	
	luxC	*luxA*
0 min	0.94 (±0.03)	1.09 (±0.06)
60 min	1.45 (±0.06)	1.55 (±0.12)
120 min	1.95 (±0.06)	2.17 (±0.03)

around 0.4 mM. This induction was not seen with strain W3110 carrying the whole plasmid, *i.e.*, pUCD615, whereas this new strain was no longer resistant to kanamycin (data not shown). Therefore, to elucidate whether this induction was due to higher transcription from the kanamycin gene, but not the origin of replication or the beta-lactamase gene, plasmid pUA5 (Fig. 1C) was constructed, and strain W3110/pUA5 was exposed to the same concentrations of benzoic acid. As with W3110/pUCD615, the bioluminescence of strain W3110/pUA5 was too low to be detected with the luminometer, *i.e.*, <0.01 A.U. (data not shown), demonstrating that the gene encoding for kanamycin resistance or its upstream region is responsible for both the basal level expression and responsiveness to benzoic acid seen with strain W3110/pUCDK. Furthermore, RT-PCR analysis of both the *luxC* and *luxA* genes showed a 2-fold increase of the mRNA levels 2 h after the exposure to 1.9 mM benzoic acid (Table 1). Both genes showed similar levels of induction, which was expected, since they are transcribed on the same mRNA molecule.

The region encoding the kanamycin gene was sequenced and submitted to the National Center for Biotechnology Information (NCBI) (Accession # AY623046). This sequence is nearly identical (~98%) with numerous other sequences listed within the NCBI nucleotide database, and shows that the protein conferring resistance to kanamycin is an aminoglycoside acetyltransferase encoded by the *aac(6')*-Ib gene.

Effect of Media pH on the Response of Strain W3110/pUCDK to Benzoic Acid

The *aac(6')*-Ib gene was previously thought to be constitutively expressed, explaining the high basal level of bioluminescence seen; however, the exact mechanism underlying induced transcription in the presence of benzoic acid remains largely unclear. One study found the induction of more than 30 proteins when *E. coli* was exposed to 20 mM benzoic acid [15]. They also reported that, of the proteins that are induced after exposure to benzoic acid, several are expressed in a pH-dependent manner. Therefore, if the responses of strain W3110/pUCDK were due to acidification of the cytosol through an uncoupling effect, they should either be diminished or absent altogether when the culture was grown in LB medium that was buffered to maintain pH 8.

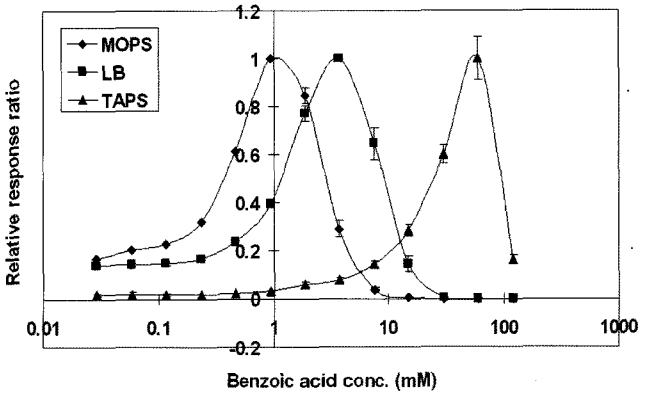

Fig. 3. pH effects on the responses from W3110/pUCDK. Bioluminescence values are shown relative to the maximum value observed for each pH tested [6.5 (MOPS), 7.2 (LB), or 8.2 (TAPS)] for a wide range of benzoic acid concentrations.
The media was buffered as described in Materials and Methods. All samples were analyzed in triplicate for error analysis, and the standard deviations are shown as error bars.

To test this, strain W3110/pUCDK was cultured at three different pHs, 6.5, 7.2, and 8.2, before being exposed to benzoic acid. Figure 3 clearly shows that, as the pH of media was increased, the response of strain W3110/pUCDK to lower concentrations of benzoic acid decreased, while leading to the greatest induction and increasing toxic responses: For instance, a concentration of 15 mM was both highly lethal and slightly responsive in the cultures grown at pH of 6.5 and 8.2, respectively, unequivocally showing the dependence of the response and benzoic acid toxicity on the pH of media. These results are in good agreement with that of Lambert *et al.* [15], who found that 20 mM benzoic acid was more potent and lethal at lower pHs. The inverse was also true, since lower pH led to a much more sensitive response from W3110/pUCDK, with the lower limit of detection (*i.e.*, RBL>2) at pH 6.5 and 8.2 being 0.05 mM and 1 mM benzoic acid, respectively. Therefore, the induction of this strain was most likely due to acidification of *E. coli*'s cytoplasm via dissociation uncoupling of the acidic moiety of benzoic acid and, therefore, it is dependent on the pH of media.

W3110/pUCDK is Responsive to Benzoic Acid Derivatives and Phenolics

To test whether dissociation can account for the responses seen, strain W3110/pUCDK was exposed to many derivatives of benzoic acid and phenol (Table 2) using unbuffered LB media. Table 2 lists the response characteristics, *i.e.*, maximum response, minimum detectable concentration, *etc.*, for each of the compounds. The results show that all but a few of the compounds led to a significant induction of the bioluminescent signal. Ironically, kanamycin was

Table 2. Names and structures of the chemicals used in this study and the response characteristics of strain W3110/pUCDK when exposed to each of them.

	Position on the benzene ring						Response characteristics				
Chemical name	C1	C2	C3	C4	C5	C6	RBL[a]	Conc. (mM)[b]	pH[c]	Time[d]	pKa
Benzoic acid (BA)	-COOH	-H	-H	-H	-H	-H	6.6	3.75	7.2	90	4.2
Salicylic acid (SA)	-COOH	-OH	-H	-H	-H	-H	3.6	2.50	7.2	90	3.0
2,5-Dihydroxy BA	-COOH	-OH	-H	-H	-OH	-H	11.1	3.13	6.8	70	
3,4-Dihydroxy BA	-COOH	-H	-OH	-OH	-H	-H	7.8	3.13	6.9	70	
3,5-Dihydroxy BA	-COOH	-H	-OH	-H	-OH	-H	9.4	3.13	6.8	70	
2-Chloro BA	-COOH	-Cl	-H	-H	-H	-H	7.8	0.39	7.2	170	2.9
3-Chloro BA	-COOH	-H	-Cl	-H	-H	-H	7.7	0.39	7.2	170	3.8
4-Chloro BA	-COOH	-H	-H	-Cl	-H	-H	12.6	0.19	7.3	170	4.0
3-Methyl SA	-COOH	-OH	$-CH_3$	-H	-H	-H	7.5	0.39	7.3	170	
4-Methyl SA	-COOH	-OH	-H	$-CH_3$	-H	-H	7.3	0.78	7.2	170	
5-Methyl SA	-COOH	-OH	-H	-H	$-CH_3$	-H	5.7	1.56	7.3	160	
4-Chloro SA	-COOH	-OH	-H	-Cl	-H	-H	8.8	0.39	7.3	150	
5-Chloro SA	-COOH	-OH	-H	-H	-Cl	-H	4.4	0.39	7.2	150	
Acetyl SA	-COOH	$-OC(O)CH_3$	-H	-H	-H	-H	10.4	0.78	7.2	150	
m-Anisic acid	-COOH	-H	$-OCH_3$	-H	-H	-H	8.3	0.78	7.2	170	4.1
p-Anisic acid	-COOH	-H	-H	$-OCH_3$	-H	-H	7.1	0.39	7.2	170	4.5
Benzylamine	$-CH_2NH_2$	-H	-H	-H	-H	-H	NR[e]	NR	8.1[f]	NR	
Benzonitrile	-CN	-H	-H	-H	-H	-H	NR	NR	7.3[f]	NR	
Phenol	-OH	-H	-H	-H	-H	-H	5.4	0.41	7.3	30	9.9
o-Cresol	-OH	$-CH_3$	-H	-H	-H	-H	4.3	2.9	7.3	50	10.3
p-Cresol	-OH	-H	-H	$-CH_3$	-H	-H	3.7	1.5	7.3	30	10.2
2-Chlorophenol	-OH	-Cl	-H	-H	-H	-H	4.4	0.02	7.3	40	8.4
2,4-Dichlorophenol	-OH	-Cl	-H	-Cl	-H	-H	2.2	0.01	7.3	50	

[a]Maximum relative bioluminescence.

[b]Concentration of the compound giving the maximum response.

[c]pH of the media when containing the concentration giving the highest response. The pH of media alone was 7.3.

[d]Time needed for the maximum relative response to be achieved.

[e]NR, No response. The RBL value is below 2 for all concentrations tested.

[f]The pH for benzonitrile and benzylamine was tested with a concentration of 3.13 mM.

one of the few chemicals that showed no induction (data not shown), a finding that is not unexpected, given the constitutive nature of the gene's promoter. Evidence that uncoupling is responsible for the induced BL can also be seen in the responses to benzonitrile and benzylamine; the structures of both are similar to benzoic acid. Since both of these compounds have nonacidic moieties on the C1 carbon (-CN and CNH_2, respectively), and thus cannot cause uncoupling of the proton motive force and a subsequent decrease in the intracellular pH, neither of these chemicals should lead to an induced BL level if uncoupling is indeed responsible. Table 2 shows that this was the case. Furthermore, the results show that this induction was not limited to benzoic acid and its derivatives, but also included other uncouplers, including numerous phenolics.

The compounds showing responses could be classified into three subgroups based on their response time, with the maximum RBL seen at around 40 min for phenolics and 80 min or 170 min for the benzoic acid derivatives. All the phenolics have high pKa values compared with those available for the benzoic acids. These higher pKa values keep the charge of the phenolics neutral at physiological pHs, which makes their transport across the membrane easier. On the other hand, the lower pKa values for the benzoic acids indicate a greater percentage of the compound in their ionized form in the media, thus hindering their transport. In addition, all of the compounds that showed a delayed maximum response are benzoic acid derivatives that have a substituted group, *i.e.*, a methyl group or chlorine, on the benzene ring. These moieties are relatively large and, in the case of the chlorine atom, have a partial charge, both of which hinder the transport of the molecule through the membrane, and the hindrance may not be as evident with the phenolics because of their neutral charge.

Induction is Not Controlled by either FadR, RpoH, or H-NS

Many of the compounds listed in Table 1 are known to induce the heat-shock response within *E. coli*. One previous study showed that the toxicity and induction of the *fabA* gene, which is regulated by the FadR protein, by different phenolics was dependent on the pKa of the

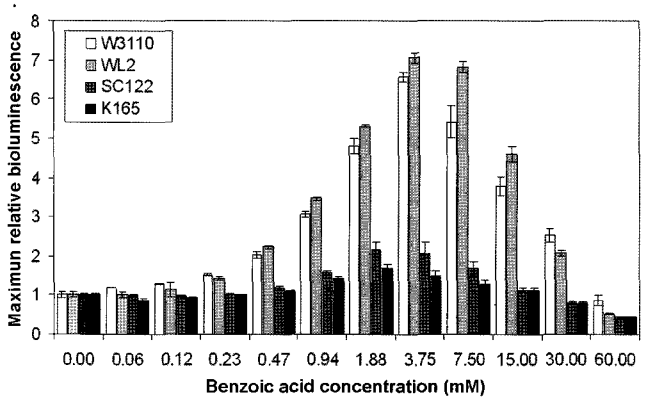

Fig. 4. Maximum relative bioluminescent responses from several *E. coli* strains when exposed to various concentrations (mM) of benzoic acid.

The pH of the media was 7.2. All samples were analyzed in triplicate for error analysis, and the standard deviations are shown as error bars.

phenol [6], indicating that the response is also dependent upon the pH of the media. Furthermore, two studies [3, 17] found that the H-NS protein plays a role in regulating the expression of the kanamycin gene.

Therefore, to determine if the induced BLs seen with strain W3110/pUCDK are mediated by the heat-shock response or fatty acid biosynthesis via the RpoH or FadR regulatory proteins, respectively, or by way of the H-NS protein, several strains of *E. coli* were transformed with plasmid pUCDK and exposed to the same concentrations of benzoic acid. As shown in Fig. 4, although the responses from strain K165 (*rpoH*)/pUCDK were fairly low, in comparison with W3110/pUCDK, with only one concentration showing a significant induction (RBL>2), its isogenic *rpoH*+ strain, SC122/pUCDK, was also just as unresponsive. Strains W3110(*wt*)/pUCDK and WL2 (*fadR*)/pUCDK responded similarly, whereas *E. coli* strain ID18/pUCDK, which has a nonfunctional *hns* gene, also showed a strong induction (data not shown). Therefore, although not entirely conclusive, these results imply that the responses from the *aac(6')*-Ib gene of plasmid pUCD615 are not regulated by the FadR protein, the RpoH sigma factor, or the H-NS protein, which are responsible for regulation of fatty acid biosynthesis, the expression of genes that aid in chaperoning, and refolding of denatured proteins during heat-shock and aiding in the structuring of the chromosome, respectively. Based on the results obtained with strains SC122 and K165, which showed reduced inducibilities, some regulatory protein would appear to exist; however, it is not clear by what mechanism it acts on the *aac(6')*-Ib promoter or gene.

Acknowledgments

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