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Use of an *ipb-lux* Fusion To Study Regulation of the Isopropylbenzene Catabolism Operon of *Pseudomonas putida* RE204 and To Detect Hydrophobic Pollutants in the Environment†

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A DNA segment involved in the regulation of the isopropylbenzene (cumene) catabolism operon (*ipb*) of plasmid pRE4 from *Pseudomonas putida* RE204 and the *Vibrio fischeri* luciferase genes, *luxCDABE*, were used to create an *ipbRo/pA′-luxCDABE* reporter fusion plasmid, pOS25. *Escherichia coli* HMS174(pOS25) produces light in the presence of inducers of the *ipb* operon. These inducers were shown to be hydrophobic compounds and to include monoalkylbenzenes, substituted benzenes and toluenes, some alkanes and cycloalkanes, chlorinated solvents, and naphthalenes. Complex hydrocarbon mixtures, such as gasoline, diesel fuel, jet fuels (JP-4 and JP-5), and creosote, were also inducers of *ipb-lux*. Bacteria carrying the *ipb-lux* reporter may be useful as bioindicators of hydrocarbon pollution in the environment and may be particularly valuable for examining the bioavailability of inducing pollutants.

The isopropylbenzene (cumene) catabolism operon (*ipb*), located on plasmid pRE4 in *Pseudomonas putida* RE204, specifies the catabolism of alkylbenzenes, such as toluene, ethylbenzene, isopropylbenzene, and *n*-butylbenzene (8), and the biotransformation of a variety of substrate analogs (5, 7). Most of the genes involved in the conversion of isopropylbenzene to isobutyrate, pyruvate, and acetyl coenzyme A, as well as an *ipbR* gene that encodes a putative positive regulatory protein, were located in a previous study by using a combination of Tn*5* mutagenesis, cloning, and restriction mapping (8). In that study, although it was possible to eliminate most pathway intermediates as inducers, it was not possible to determine whether isopropylbenzene or the first intermediate in the pathway, 2,3-dihydroxy-2,3-dihydroisopropylbenzene, is the inducer. To unequivocally identify isopropylbenzene and other alkylbenzenes that serve as growth substrates as inducers, it is necessary to have a strain which does not transform alkylbenzenes and in which the regulatory elements of the *ipb* operon are upstream of a gene(s) that encodes an assayable enzyme. Tn*5* mutants that do not metabolize alkylbenzenes were available; however, the polarity of the Tn*5* insertions into the isopropylbenzene dioxygenase genes also prevented the inducible expression of assayable enzymes encoded by downstream genes. In order to study regulation of the *ipb* operon, plasmid pOS25 was constructed. This plasmid contains the regulatory elements of the *ipb* operon, *ipbR* and *ipbo/p*, upstream of promoterless luciferase genes *luxCDABE* from *Vibrio fischeri*. The genes *luxA* and *luxB* encode α and β subunits of the enzyme luciferase, which converts a long-chain aldehyde substrate to the corresponding acid with emission of light (25), while *luxC*, *luxD*, and *luxE* encode a reductase, a transferase, and a synthetase, respectively, which constitute the fatty acid reductase complex responsible for the synthesis of the luciferase substrate.

These luciferase genes have been used in the past in plasmid constructions as highly sensitive reporters for detecting specific chemicals, such as naphthalene and salicylate (15), and mercuric ions (26), and for monitoring stresses caused by various environmental contaminants (35). Our expectation was that *lux*-specified light production by bacteria carrying pOS25 would be inducible by chemicals that induce *ipb* expression without the possibility that those chemical inducers would be metabolized. When we used a bioreporter strain, *Escherichia coli* HMS174(pOS25), it was possible to identify not only inducers of the *ipb* operon that serve as growth substrates but also a variety of gratuitous inducers. Some possible applications of the *ipb* regulatory system and *ipb-lux* fusions based on these results are described in this paper.

(This work has been presented in a preliminary form previously [27, 29].)

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. *P. putida* RE204 (8), which grows with isopropylbenzene and other alkylbenzenes as sole sources of carbon and energy, and *E. coli* HMS174 [*F⁻recA1 rpoB331 hsdR19* λ⁻ IN (*rrnD-rrnE*)*1*] (4) were used in this study. The minimal medium used for *E. coli* HMS 174 was R medium (6) supplemented with 10 trace elements (10), 10 mM pyruvic acid, 0.05% glycerol, and 50 mg of kanamycin per liter (M medium), and the minimal medium used for *P. putida* RE204 was R medium supplemented with 0.1% succinate and 0.05% yeast extract. Luria-Bertani medium and agar plates were prepared as described by Miller (18).

Restriction endonucleases, T4 DNA ligase, and β-agarase were obtained from New England Biolabs, Beverly, Mass., and were used as suggested by the manufacturer. Benzaldehyde was obtained from Fisher Scientific Co., Norcross, Ga.; benzoic acid was obtained from Sigma Chemical Co., St. Louis, Mo.; cyclohexane and hexane were obtained from J. T. Baker, Inc., Phillipsburg, N.J.; iodobenzene was obtained from Eastman Kodak Co., Rochester, N.Y.; pentachloroethane was obtained from Lancaster Synthesis, Inc., Windham, N.H.; phenol was obtained from Gibco BRL, Grand Island, N.Y.; and all other individual chemicals were

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TABLE 1. Induction of luciferase expression by benzene, alkylbenzenes, and isopropylbenzene metabolites

Chemical	Minimal induction[a]			Maximal induction[b]		
	Concn (μM)	Ratio after[c]:		Concn (μM)	Ratio after[c]:	
		100 min	250 min		100 min	250 min
Benzene	10	2	2.5	1,000	66	4,100
Toluene	1	2.5	6	100	21	2,300[d]
Ethylbenzene	1	5	28	100	14	4,800
Isopropylbenzene	1	2	2	100	5	4,900
n-Butylbenzene	100	4	9	500	6	25
n-Amylbenzene	100	1.5	1.5	1,000	1.5	1.5
3-Isopropylcatechol	10	2	3	100	3.5	11
2,3-Dihydroxy-2,3-dihydro-isopropylbenzene		NR[e]	NR		NR	NR

[a] The level of luciferase activity at the lowest concentration detected.
[b] The highest luciferase activity detected.
[c] Ratio of induced activity to uninduced activity.
[d] The maximal level of induction by toluene was 4,800-fold after 200 min; this value decreased to 2,300-fold by 250 min.
[e] NR, no response.

obtained from Aldrich Chemical Co., Milwaukee, Wis. 3-Isopropylcatechol was recrystallized from pentane. 2,3-Dihydroxy-2,3-dihydroisopropylbenzene was prepared by incubating mutant strain *P. putida* RE213 (8) with isopropylbenzene. The dihydrodiol product was extracted with ethyl acetate and purified by chromatography on Sephadex LH-20 with ethanol as the solvent, as previously described (5). Creosote was obtained from Aristech Chemical Corp., Clairton, Pa. JP-4 and JP-5 jet fuels were generously provided by J. Spain (Tyndall Air Force Base, Fla.) and R. Bell (Naval Air Station, Pensacola, Fla.), respectively. Gasoline (87 octane) and diesel fuel were obtained at an Exxon gasoline station in Pensacola, Fla.

Construction of pOS25. Plasmid pOS25 (Fig. 1) carries the regulatory elements of the isopropylbenzene catabolism operon (*ipb*) from pRE4 of *P. putida* RE204 (8) upstream of luciferase (*lux*) genes from *V. fischeri* (25). This plasmid was constructed by using conventional methods (16), as follows. Plasmid pRE61 carries a 9.8-kb *Bam*HI fragment from pRE4 with genes encoding isopropylbenzene dioxygenase (*ipbA*) downstream from an operator-promoter (*ipbo/p*) and a gene encoding a regulatory protein (*ipbR*). A 5.8-kb *Bgl*II-*Apa*I fragment from pRE61 was inserted into *Bam*HI-*Apa*I-digested pUC18-derived vector pUCBM20 (Boehringer-Mannheim Corp., Indianapolis, Ind.) (37), which yielded pOS24. The DNA sequence of this *Bgl*II-*Apa*I fragment has been determined, and this sequence confirmed the locations of the *ipb* genetic elements identified previously (8, 28). Complete digestion of pOS24 with *Xba*I and partial digestion with *Eco*RI produced a mixture of fragments that included a 5.8-kb *Xba*I-*Eco*RI fragment carrying *ipbRo/pA′*. Plasmid pRB28 (26) carries promoterless *luxCDABE* genes downstream from *merRo/pT′* (mercury resistance operon regulatory sequences). pRB28 was digested with *Xba*I and *Eco*RI, and the 17.5-kb fragment was separated from the 0.7-kb *mer* gene-carrying fragment by electrophoresis through low-melting-temperature agarose; the 17.5-kb DNA fragment was recovered from the gel by using β-agarase. *Eco*RI-*Xba*I fragments from pOS24 and pRB28 were mixed and ligated, and the mixture was used to transform *E. coli* HMS174. Transformants were selected on Luria-Bertani agar plates containing 50 μg of kanamycin per ml. Light-producing colonies were readily identified in the dark.

Luciferase induction assays. Fresh colonies of *E. coli* HMS174(pOS25) on agar plates containing equal parts of Luria-Bertani medium and M medium were used to inoculate 5 ml of M medium; after overnight incubation at 30°C, the culture was diluted with 5 volumes of M medium in a 125-ml Erlenmeyer flask, and the resulting preparation was incubated at 30°C with shaking until it reached an optical density at 660 nm of 0.1. Cells were harvested by centrifugation (2,500 rpm for 8 min in a Sorvall model RT6000B refrigerated centrifuge at 22°C), resuspended in the same volume of M medium, and used immediately. Strain HMS174(pOS25) was exposed to individual chemicals at concentrations of 0.1, 1, 10, 100, and 500 μM and 1 mM or chemical mixtures at concentrations of 0.01, 0.1, 1, 10, 50, and 100 ppm in M medium. The stock solutions of chemicals tested as inducers were prepared in ethanol, and 2-μl portions of appropriate dilutions were added to 1-ml portions of a cell suspension. Assays of luciferase-catalyzed light production were performed as previously described (26) by using a Packard model 2500 Tri-Carb TR liquid scintillation counter. Luminescence was expressed as quanta per second per milliliter by transforming photon-per-minute data by using the Hastings-Weber standard (12). The results presented below are the averages of the values obtained in two to four independent analyses. Induction is expressed as the ratio of the amount of light produced by induced cells to the amount of light produced by uninduced (ethanol only) cells.

Induction assays performed with *P. putida* RE204. Overnight cultures of *P. putida* RE204 in 50 ml of R medium supplemented with 0.1% succinate and 0.05% yeast extract were used to inoculate 500-ml portions of R medium containing 0.1% succinate and 0.02% yeast extract. The resulting cultures were incubated at 30°C for 6 h with and without inducers added to the medium after 2 and 4 h. Isopropylbenzene, tetrachloroethylene, and trichloroethylene were added to a concentration of 500 μM, and naphthalene was added to a concentration of 100 μM. Cells were harvested by centrifugation, washed once with 30 ml of 50 mM KH_2PO_4–NaOH buffer (pH 7.5), and resuspended in 4.5 ml of the same buffer. Extracts were prepared by passing the cell suspensions twice through a French pressure cell at between 14,000 and 20,000 lb/in^2 at 4°C and then centrifuging the preparations at 20,000 rpm in a Sorvall type SS-34 rotor for 40 min. Protein concentrations were determined by using the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, Ill.) with bovine serum albumin as the standard.

Induction of the *ipb* operon was measured by performing an assay for a representative enzyme, 3-isopropylcatechol 2,3-dioxygenase. The assay was performed by adding 5 to 15 μl of cell extract to a spectrophotometer cuvette containing 985 to 995 μl of 200 μM 3-isopropylcatechol and 50 mM KH_2PO_4–NaOH buffer (pH 7.5). Formation of the ring cleavage product 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate was monitored by recording the increase in A_{393} (ε_{393} = 9,700 M^{-1} cm^{-1}) over time.

Preparation of sediment and soil samples. Sediment samples that were collected in November 1993 from the South Chesapeake Bay area of Wicomico County, Md., were obtained from Mitchell Brourman, Beazer East, Inc., and were kept at 4°C in closed jars. Noncontaminated soil samples were collected in November 1994 on Sabine Island, Pensacola Beach, Fla. To extract ethanol-soluble chemicals, 5-g portions of sediment or soil samples were suspended in 5-ml portions of absolute ethanol and shaken for 16 to 18 h in 25-ml glass flasks with Teflon-lined screw caps. Each suspension was centrifuged for 10 min at 3,500 rpm in a Sorvall model RT600B centrifuge. The upper phase (approximately 5 ml) was removed, filtered through a disposable syringe nylon filter (diameter, 25 mm; Corning, Houston, Tex.), and used for gas chromatography-mass spectrometry analysis and *ipb-lux* induction assays.

Chemical analyses. Gas chromatography-mass spectrometry analyses were performed with a Hewlett-Packard model 5890 Series II gas chromatography equipped with a model 5971A mass-selective detector. Helium at a linear velocity of 24 cm/s was used as the carrier gas. A 1-μl Chesapeake Bay sediment extract sample was injected into a type HP-5 capillary column (25 m by 0.32 mm; film thickness, 0.52 μm; Supelco, Bellefonte, Pa.). The oven temperature was kept at 45°C for 3 min and then gradually increased at a rate of 5°C/min to 280°C. It was kept at 280°C for 5 min, after which it was increased to 310°C at a rate of 3°C/min, where it remained for 10 min. Chemical concentrations were calculated by using external standards consisting of the chemicals used.

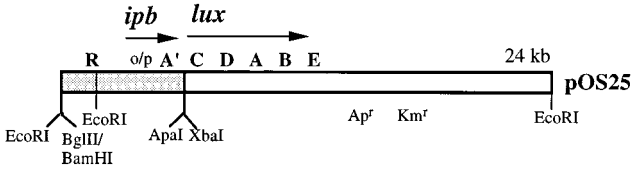

FIG. 1. *ipb-lux* fusion plasmid pOS25. pOS25 contains a *Bgl*II-*Apa*I fragment (coordinates 21 to 15.3) (8) of the *ipb* operon carrying *ipbR*, o/p, and *A′*, as well as promoterless *luxCDABE* from *V. fischeri* (25). The arrows indicate the direction of transcription.

TABLE 2. Induction of luciferase expression by aromatic and nonaromatic chemicals[a]

Chemical[b]	Minimal induction			Maximal induction		
	Concn (μM)	Ratio after:		Concn (μM)	Ratio after:	
		100 min	250 min		100 min	250 min
Halogen- and alkyl-substituted benzenes and toluenes						
Fluorobenzene	10	3	4	1,000	20	1,000
Chlorobenzene	1	4	12	100	16	1,200
Iodobenzene	0.1	4.5	20	10	10	600
α,α,α-Trifluorotoluene	1	2	2.5	500	15	510
2-Chlorotoluene	0.1	2	3	10	4	900
2-Bromotoluene	0.1	3	4.5	10	5	200
2-Iodotoluene	0.1	3	7	1	5	90
p-Xylene	1	2	2	100	11	2,000
1,2-Diethylbenzene	1	1.5	2	100	4	250
1,2,4-Trimethylbenzene	10	4	21	100	5.5	480
1,2,3,4-Tetramethylbenzene	10	3	9	100	5	140
1,2,4,5-Tetramethylbenzene	100	3	4.5	1,000	4.5	9.5
p-Cymene	100	2	2.5	500	2.5	5.5
Naphthalenes						
Naphthalene	0.1	3.5	15	10	7	1,700
1-Methylnaphthalene	1	4.5	13	10	5	120
2-Methylnaphthalene	1	1.5	2	100	11	1,100
1-Ethylnaphthalene	1	2	3	100	5.5	98
Other aromatic chemicals						
Benzothiophene	0.1	3	7	10	5	67
Indan	1	5	25	10	5	2,900
1-Indanone	10	3.5	4	500	19	2,800
Indole	10	6	7	100	11	45
Aniline	100	1.5	1.5	1,000	4	8
Pyridine	500	1.5	1.5	1,000	2	2
Benzaldehyde	10	2.5	2.5	500	12	130
p-Tolualdehyde	10	4.5	3.5	100	12	30
Benzyl alcohol	100	2	2	1,000	6	11
Phenol	100	2	2	1,000	3.5	4
Nonaromatic chemicals						
Hexane	100	3.5	3.5	1,000	6	590
Cyclohexane	100	3	4	1,000	6	1,000
Methylcyclohexane	100	3.5	5	1,000	6	690
Ethylcyclohexane	100	4	12	1,000	6.5	470
Decalin	10	2	2	500	5	250
Cyclohexene	10	2	2	500	5	340
Trichloroethylene	10	2	2	500	28	1,450
Tetrachloroethylene	1	3.5	6.5	100	10	3,700
Tribromoethylene	0.1	3	6	10	10	840
Pentachloroethane	1	2	3	10	4.5	35
1,1,2,2-Tetrachloroethane	1	2	2	100	9	250
1,2-Dichloroethane	500	2	2	1,000	3	4

[a] See the footnotes to Table 1.
[b] There was no response to the following chemicals: *n*-butane, decane, hexadecane, benzoic acid, protocatechuic acid, biphenyl, 2-ethylnaphthalene, 9-fluorenone, and dibenzofuran.

RESULTS AND DISCUSSION

Plasmid pOS25 was constructed by fusing regulatory region *ipbRo/pA′* of the isopropylbenzene catabolic operon of pRE4 upstream of *luxCDABE* genes from *V. fischeri* (Fig. 1). *E. coli* HMS174 carrying this plasmid was then used to study regulation of the *ipb* operon. After chemical inducers were added to *E. coli* HMS174(pOS25) cells, the resulting luciferase-catalyzed light production was recorded over time with a scintillation counter.

Initially, isopropylbenzene, other alkylbenzenes, and the early pathway intermediates 2,3-dihydroxy-2,3-dihydroisopropylbenzene and 3-isopropylcatechol were examined as inducers (Table 1). The aromatic hydrocarbon isopropylbenzene, as well as other alkylbenzenes that are growth substrates for *P. putida* RE204 using the enzymes of this pathway, are clearly very good inducers, while the more polar pathway intermediates are not.

In order to determine the range of chemicals able to induce the *ipb* operon, additional chemicals were tested as inducers of

TABLE 3. Enzyme activities in cell extracts of *P. putida* RE204

Inducer	Sp act of 3-isopropylcatechol dioxygenase (nmol min^{-1} mg of $protein^{-1}$)
None	9
Isopropylbenzene	1,220
Naphthalene	1,360
Tetrachloroethylene	1,020
Trichloroethylene	450

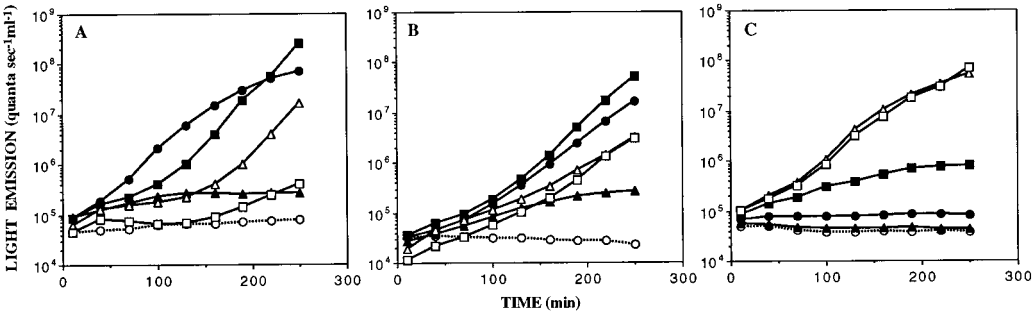

FIG. 2. Luciferase expression in response to the presence of isopropylbenzene (A), naphthalene (B), and trichloroethylene (C). (A and C) Symbols: ○, no chemical; ▲, 1 μM; ●, 10 μM; ■, 100 μM; △, 500 μM; □, 1,000 μM. (B) Symbols: ○, no chemical; ▲, 0.1 μM; ●, 1 μM; ■, 10 μM; △, 100 μM; □, 500 μM.

luciferase activity in strain HMS174(pOS25); many of these chemicals proved to be effective (Table 2). In general, the good inducers were hydrophobic compounds; several of the polar compounds tested, including benzoate, aniline, pyridine, benzyl alcohol, and phenol, were poor inducers or noninducers. Although aromaticity is not a requirement for chemicals to act as inducers, cyclohexane, methylcyclohexane, ethylcyclohexane, and decalin produced maximum levels of induction that were severalfold lower than the levels produced by the corresponding aromatic compounds (benzene, toluene, ethylbenzene, and naphthalene). The size of the compound was important; aromatic compounds having one small substituent were better inducers than aromatic compounds having several substituents or large substituents. Several of the volatile halogenated solvents tested, especially tetrachloroethylene (perchloroethylene) and trichloroethylene, were effective inducers. Again, the size of the chemical was important; the largest volatile halogenated solvent, pentachloroethane, and the smallest, 1,2-dichloroethane, were poor inducers.

Heald and Jenkins (14) demonstrated that trichloroethylene can induce the toluene catabolic pathway in *P. putida* to a level that is about 40% of the level attained when toluene is the inducer. This level is very similar to the level obtained with *E. coli* HMS174(pOS25), in which the level of induction by trichloroethylene is about 30% of the level of induction by isopropylbenzene or toluene. More recently, McClay et al. (17) showed that toluene oxidation activity in *Pseudomonas mendocina* KR1 or *Pseudomonas* sp. strain ENVPC5 can be induced by trichloroethylene to a level that is as high as 86% of the level obtained with toluene-induced cells. Other chlorinated solvents and alkanes also served as inducers.

Regulatory proteins that control expression of other aromatic compound catabolic pathways have been shown to have relaxed specificity. The regulator of the TOL plasmid-encoded upper pathway which transforms toluenes to benzoates, the XylR protein, is activated not only by toluenes and xylenes, but also by various substituted toluenes, benzyl alcohols, and *p*-chlorobenzaldehyde (1). Another protein, DmpR, the regulator of the operon encoding dimethylphenol metabolism, *dmp*, also responds to a wide range of aromatic compounds, mostly various substituted phenols (24, 30). Nevertheless, so far, both XylR and DmpR have been tested with a fairly limited spectrum of structurally related compounds.

The level of *ipb-lux* induction attained is related to inducer concentrations (Fig. 2). Thus, the response to isopropylbenzene increased as the concentration of this compound was increased from 1 to 100 μM (Fig. 2A). At higher concentrations, the level of induction decreased (by as much as 99% at 1 mM isopropylbenzene). This decrease may have been due to the toxicity of this and other hydrocarbons to bacterial cells due to alterations of membrane structure and function (31). Naphthalene induced *ipb-lux* at a concentration of 0.1 μM (Fig. 2B); increasing the concentration yielded higher levels of induction up to a concentration of 10 μM. However, at a concentration of 100 μM, naphthalene began to precipitate out of solution and the level of induction decreased. This illustrates one of the practical limitations for application of this system: hydrophobic compounds are inducers, but at high concentrations they are not water soluble. Trichloroethylene did not appear to present problems of toxicity or solubility at the concentrations which we tested (Fig. 2C).

Analysis of induced enzymatic activity in *P. putida* RE204. On the basis of the results obtained with the *ipb-lux* reporter, we reexamined induction of the *ipb* operon in *P. putida* RE204 with compounds that had been shown to induce *ipb-lux* well. Extracts of strain RE204 grown in the presence of isopropylbenzene, naphthalene, tetrachloroethylene, and trichloroethylene were assayed for activity of a representative enzyme of the isopropylbenzene pathway, 3-isopropylcatechol dioxygenase (Table 3). As we found with *E. coli* HMS174(pOS25), all of these compounds induced high levels of the enzyme assayed.

Induction of *ipb-lux*-regulated luciferase expression generally became detectable 1 h after an inducer was added. About 4 h was required for the maximum level of induced light production. This slow development of the bioluminescent response in *E. coli* HMS174(pOS25) may reflect the time required for hydrophobic chemicals to enter gram-negative bacterial cells across the outer membrane barrier (22). Little is known about the specific transport of aromatic hydrocarbons into bacterial cells, although it is generally assumed that these

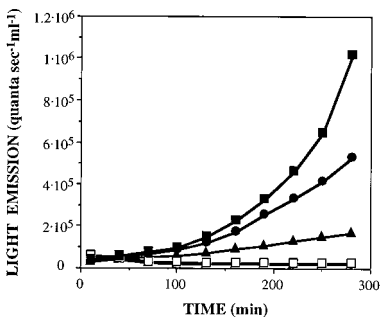

FIG. 3. Bioluminescent response of strain HMS174(pOS25) in the presence of ethanol extracts from Chesapeake Bay sediments at dilutions of 1:500 (▲), 1:200 (●), and 1:100 (■). □, uninduced cells.

TABLE 4. Induction of luciferase expression by hydrocarbon mixtures[a]

Chemical	Minimal induction			Maximal induction		
	Concn (ppm)	Ratio after:		Concn (ppm)	Ratio after:	
		100 min	250 min		100 min	250 min
Gasoline	0.1	2	2	50	6	45
Jet fuel JP-4	1	3	4.5	100	6	45
Jet fuel JP-5	1	1.5	2	100	4	30
Diesel fuel	1	1.5	2	100	4	29
Creosote	0.01	9	35	0.1	19	130

[a] See the footnotes to Table 1.

compounds enter cells that degrade them without the aid of active transport mechanisms. A recent study of the *tod* operon, which specifies toluene degradation in *P. putida* F1, demonstrated the presence of a gene encoding a putative membrane protein (TodX) which was proposed to function in toluene transport (36). However, a transport protein(s) associated with the *ipb* operon has not been demonstrated.

While the most important property of a compound that allows it to induce *ipb-lux* appears to be hydrophobicity, other properties of hydrophobic compounds, particularly their toxicities to cells and limited water solubilities, decrease their value as inducers of *ipb-lux*.

Induction of luciferase by hydrocarbon mixtures. The suitability of the *ipbRo/pA'-luxCDABE* reporter for detecting hydrocarbon mixtures, including petroleum-derived products and coal tar creosote, was tested. Petroleum products, such as gasoline, jet fuels (JP-4 and JP-5), and diesel fuel, are mixtures of hundreds of chemicals, primarily aliphatic hydrocarbons (paraffins, olefins, and naphthenes [60 to 80%]) and aromatic compounds. Gasoline is a mixture of C_4 to C_{12} hydrocarbons that boil at temperatures between 39 and 204°C; jet fuels (JP-4 and JP-5) contain C_8 to C_{15} hydrocarbons that have a boiling temperature range of 180 to 280°C, while diesel fuel includes C_{10} to C_{22} hydrocarbons that boil at temperatures between 190 and 380°C (3, 9, 13, 21, 23, 32, 33). Coal tar creosote is composed of 150 to 200 chemicals and consists of about 85% polycyclic aromatic hydrocarbons, 10% phenolic compounds, and 5% N-, S-, and O-heterocyclic compounds. Naphthalene and substituted naphthalenes account for 30 to 40% of creosote (19, 20).

All of the mixtures mentioned above induced *ipb-lux* to some extent (Table 4). The response to creosote was greatest and also occurred at the lowest concentration of any mixture (0.01 ppm). This may be a result of the higher concentration of aromatic compounds in creosote than in the other mixtures. The aromatic compounds, particularly the phenolic compounds, may also be responsible for the increased toxicity of creosote at relatively low concentrations. While the ability of a pure chemical to induce is related to a combination of factors, we expect that the abilities of chemical mixtures to act as inducers are also related to an even more complex combination of relative concentrations of inducers and inhibitors, the toxicities of all of the components of the mixtures, and the solubilities of the components.

Analyses of hydrocarbon extracts from Chesapeake Bay sediments. Hundreds of toxic organic compounds have been identified in Chesapeake Bay sediments and in the organisms that live there, such as oysters and crabs (2, 11). Hydrocarbons enter the Chesapeake Bay from many sources, including burning of fossil fuels and wood, liquefication and gasification of coal, spillage of creosote and other wood preservatives, and atmospheric transport from distant regions by Northern Hemisphere air currents (34). Ethanol extraction of sediments from Wicomico County, Md. (South Chesapeake Bay area) yielded a solution containing a variety of polycyclic aromatic chemicals, including naphthalene, acenaphthene, dibenzofuran, fluorene, dibenzothiophene, phenanthrene, anthracene, fluoranthene, pyrene, and chrysene and their homologs (Table 5). The sediment extracts looked much like coal tar creosote (19).

Ethanol extracts of sediment and noncontaminated soil (2, 5, and 10 μl) were used in the *ipb-lux* assays with 1 ml of a cell suspension in MM medium. Ethanol extracts of noncontaminated soil did not induce light production. As increasing amounts of contaminated sediment extracts were added to the *E. coli* HMS174(pOS25) cell suspension, the bioluminescent response increased proportionately (Fig. 3). Even at the lowest concentration, the response was eightfold greater than the response obtained with uninduced cells.

This study was initiated to study the regulation of the isopropylbenzene catabolism operon and then expanded to include a study of inducer specificity and to determine whether the broad inducer specificity demonstrated might have other applications. While the *E. coli* HMS174(pOS25) bioindicator strain will not replace methods such as gas chromatography for analysis of polycyclic aromatic chemicals in the environment, it may have potential use for studying the bioavailability of a variety of polycyclic aromatic chemicals and other hydrophobic compounds since, in order to induce *ipb-lux*, these compounds must be able to enter cells (and therefore be bioavailable). A potential use for *ipbRo/p* suggested by our results is as a regulatory unit placed in recombinant genetic constructions upstream of genes whose regulation by hydrophobic inducers would be desirable.

TABLE 5. Predominant polycyclic aromatic compounds in ethanol extracts of a Chesapeake Bay sediment

Compound[a] and elution time	Concn (ppm)
Naphthalene (16.44)	12.80
1-Methylnaphthalene (19.78)	45.35
2-Methylnaphthalene (20.27)	29.20
Acenaphthene (25.05)	67.98
Dibenzofuran (25.86)	46.13
Fluorene (27.56)	55.84
Phenanthrene (32.24)	114.05
Fluoranthene (38.15)	71.74
Pyrene (39.18)	46.38
Chrysene (45.25)	7.86

[a] Compounds are listed in order of elution during gas chromatography.

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