Applied and Environmental Microbiology, Oct. 1997, p. 4053–4060 Vol. 63, No. 10
0099-2240/97/$04.00+0
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Development and Characterization of a Whole-Cell Bioluminescent Sensor for Bioavailable Middle-Chain Alkanes in Contaminated Groundwater Samples

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Received 6 May 1997/Accepted 5 August 1997

A microbial whole-cell biosensor was developed, and its potential to measure water-dissolved concentrations of middle-chain-length alkanes and some related compounds by bioluminescence was characterized. The biosensor strain *Escherichia coli* DH5α(pGEc74, pJAMA7) carried the regulatory gene *alkS* from *Pseudomonas oleovorans* and a transcriptional fusion of P_{alkB} from the same strain with the promoterless luciferase *luxAB* genes from *Vibrio harveyi* on two separately introduced plasmids. In standardized assays, the biosensor cells were readily inducible with octane, a typical inducer of the *alk* system. Light emission after induction periods of more than 15 min correlated well with octane concentration. In well-defined aqueous samples, there was a linear relationship between light output and octane concentrations between 24 and 100 nM. The biosensor responded to middle-chain-length alkanes but not to alicyclic or aromatic compounds. In order to test its applicability for analyzing environmentally relevant samples, the biosensor was used to detect the bioavailable concentration of alkanes in heating oil-contaminated groundwater samples. By the extrapolation of calibrated light output data to low octane concentrations with a hyperbolic function, a total inducer concentration of about 3 nM in octane equivalents was estimated. The whole-cell biosensor tended to underestimate the alkane concentration in the groundwater samples by about 25%, possibly because of the presence of unknown inhibitors. This was corrected for by spiking the samples with a known amount of an octane standard. Biosensor measurements of alkane concentrations were further verified by comparing them with the results of chemical analyses.

Although many contaminants are readily biodegradable, they often persist in the environment because they are degraded at rates too slow for efficient cleanup. One major factor that limits biodegradation in the environment is the insufficient accessibility of pollutants to microbial attack. This is especially true of hydrophobic compounds, such as those occurring in diesel oil contaminations. Diesel oil consists mostly of linear and branched alkanes with different chain lengths and contains a variety of aromatic compounds. Many of these compounds, especially linear alkanes, are known to be very biodegradable (35). Due to low water solubility, however, the biodegradation of these compounds is often limited by slow rates of dissolution, desorption, or transport (12, 24). In general, the bioavailability of hydrophobic compounds is determined by their sorption characteristics (12, 13, 22, 23, 25) and dissolution or partitioning rates (8, 13, 32, 33) and by transport processes to the microbial cell (12, 14).

Microorganisms themselves can be used as specific and sensitive devices for sensing the bioavailability of a particular pollutant or pollutant class. This is based on the ability of pollutants (like that of most "normal" compounds) to invoke nonspecific (e.g., toxicity or stress) or specific (e.g., activation of a degradative pathway) responses in microorganisms. The signalling pathway thus activated will regulate the expression of one or more (sets of) genes. The extent of this gene expression serves as a measure of the available ("sensed") concentration of the compound.

A rapid and sensitive way to measure such gene expression is to fuse relevant promoter sequences and promoterless reporter genes such as those for bacterial luciferases of *Vibrio* spp. (11) (for recent reviews, see references 30 and 31). Researchers who have used bacterial luciferase as a reporter gene have applied either the complete *luxCDABE* operon or the *luxAB* genes only for the two subunits of luciferase. Fusions with *luxAB* require the addition of a long-chain aliphatic aldehyde, preferably decanal, as a substrate for the luciferase reaction, whereas *luxCDABE* fusions intrinsically produce and regenerate the aldehyde substrate. The use of microbes to sense and report the presence of chemical compounds has recently provoked great interest. Whole-cell biosensors that can detect naphthalene and salicylate (15, 16), toluene (3), and mercury (27) have been developed. A fusion of the *lux* genes and the regulatory elements of the isopropylbenzene catabolism operon was used to detect various hydrophobic pollutants, such as alkylbenzenes and several other aromatic and aliphatic hydrocarbons (28). Other biosensor strains were constructed to detect toxic compounds, generally by coupling the *lux* genes with a stress-inducible promoter (34).

Unfortunately, the light emission signal measured by whole-cell biosensors is dependent not only on the available concentration of the inducing substance but also on the stability of the luciferase in the particular strain (21), the strain's physiological state (16), and the presence of other stimulating or inhibitory substances in the sample to be measured (3). The stability of the signal and the physiological state of the biosensor cells can be reasonably well controlled by optimized and standardized

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TABLE 1. Plasmids used in this work

Plasmid	Relevant characteristic(s)[a]	Source or reference
pGEM7Zf(+)	Ap^r	Promega
pUC18Not	MCS flanked by two *Not*I sites, Ap^r	17
pKK232-8	pBR322 derivative containing the *rrnB* ribosomal RNA T1 terminator, Ap^r	2
pHG171-luxAB	promoter-probe vector based on the *luxAB* genes, Ap^r	J. Kuhn
pGEc47	pLAFR1 (RK2) with *alkBFGHJKL*/*alkST*, Ap^r Tc^r	10
pGEc74	pLAFR1 (RK2) with *alkST*, Ap^r Tc^r	9
pJAMA1	pGEM7Zf(+) carrying 0.6-kb P_{alkB} PCR fragment, Ap^r	This work
pJAMA2	pHG171-luxAB carrying the 0.6-kb *Hin*dIII-*Xba*I fragment of pJAMA1, Ap^r	This work
pJAMA4	pJAMA2 carrying the 0.2-kb *Eco*RI fragment of pKK232-8, Ap^r	This work
pJAMA7	pUC18Not carrying the 3.0-kb *Hin*dIII-*Bgl*II fragment of pJAMA4, Ap^r	This work

[a] MCS, multiple cloning site.

assay conditions. Sample composition is more difficult to control and can lead to false-positive or false-negative measurements (3). Misinterpretation of false-positive results, i.e., partial or full induction of the reporter without its cognate inducer, can be avoided by a clear understanding of the biosensor specificity and knowledge of other potential inducers in typical pollutant mixtures. False-negative results, i.e., partial or full inhibition of the signal expected from a particular concentration of inducer, are mainly due to toxic compounds in the sample. This type of result can be verified by suitable control experiments, such as spiking samples with a known amount of cognate inducer (3) or applying an additional, constitutively expressed reporter system to control for the biosensor activity (16, 36).

In the present study, we describe the construction of an *E. coli* whole-cell biosensor for the detection of linear alkanes by the creation of a transcriptional fusion between the *alkB* promoter of *Pseudomonas oleovorans* and the promoterless *luxAB* genes of *Vibrio harveyi*. The strain also contained the gene for AlkS, which is the transcriptional activator of the *alkB* promoter (9). The ability of the biosensor to detect available concentrations of octane was carefully analyzed by optimized and standardized assays. The response of the sensor to other possible inducers and potential inhibitors was also tested. Finally, we describe the use of the sensor for measuring bioavailable concentrations of linear alkanes in oil-contaminated groundwater samples. The feasibility of using such strains to analyze the bioavailability of pollutants in the environment is discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* DH5α (26) was used for routine cloning experiments with the plasmids listed in Table 1. Plasmid pHG171-luxAB was the source for the *luxAB* genes of *V. harveyi* (5, 19). Upstream of the *luxAB* genes are multiple cloning sites, stop codons in all three reading frames, and a ribosome binding site (Fig. 1). Downstream lies a stem-and-loop structure which is supposed to stabilize the mRNA after transcription.

The DNA fragment with the *alkB* promoter (P_{alkB}) was obtained by PCR with plasmid pGEc47 as template DNA and primers ALKB1 (5′-GGCGTTGGTACCCCGGCTGCTTCG-3′) and ALKB2 (5′-TTTATCTACGTCGACTGGAGCGGAATCC-3′). The reaction was performed as suggested by the supplier (Gibco BRL, Life Technologies, Inc., Gaithersburg, Md.). In this way, a 0.6-kb fragment with the promoter region and a small part of the open reading frame (*alkB′*) of the *alkB* gene was obtained, flanked by a *Kpn*I and an *Sal*I site. The authenticity of this fragment was confirmed by sequencing with IRD-41-labeled primers (MWG Biotech, Ebersberg, Germany) in a Thermosequenase reaction (Amersham International plc., Little Chalfont, United Kingdom) and separating and analyzing it on an automated sequencer (model 400L; LiCOR, Lincoln, Nebr.). Next, the fragment was cut by *Kpn*I and *Sal*I, inserted into pGEM7Zf(+), and digested with *Kpn*I and *Xho*I, resulting in plasmid pJAMA1. Plasmid pJAMA2 was obtained by cloning the 0.6-kb *Hin*dIII-*Xba*I fragment with P_{alkB} from pJAMA1 into plasmid pHG171-luxAB. To prevent possible transcription from promoters located further upstream, a transcription terminator was cloned upstream of P_{alkB}. Therefore, a 0.2-kb *Eco*RI fragment containing the *rrnB* ribosomal RNA T1 terminator was isolated from plasmid pKK232-8. The fragment's 3′ recessive ends were filled in with the Klenow fragment of DNA polymerase I. Subsequently, this fragment was inserted into pJAMA2, which was first partially digested with *Asp*718I and then subjected to Klenow polymerase treatment. This resulted in plasmid pJAMA4. The whole P_{alkB}-*luxAB* fusion was then recovered from pJAMA4 as a 3.0-kb *Hin*dIII-*Bgl*II fragment and cloned into pUC18Not which was digested with *Hin*dIII and *Bam*HI. The resulting plasmid is referred to as pJAMA7 (Fig. 1). Plasmids pJAMA7 and pGEc74 were cotransformed in *E. coli* DH5α and could be stably maintained, since both plasmids are from different incompatibility groups.

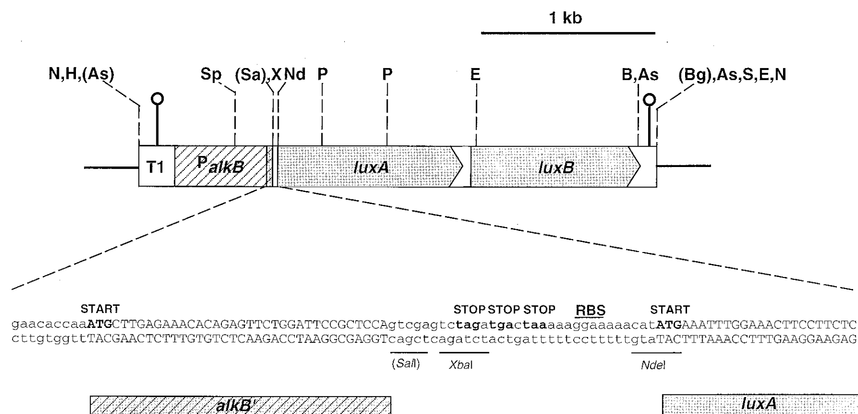

FIG. 1. Organization of the 3.0-kb *Not*I insert of pJAMA7. The remaining coding sequence of *alkB* is indicated with *alkB′*. Abbreviations: As, *Asp*718I; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; N, *Not*I; Nd, *Nde*I; P, *Pst*I; S, *Sst*II; Sa, *Sal*I; Sp, *Sph*I; X, *Xba*I; T1, *rrnB* ribosomal RNA T1 terminator; P_{alkB}, promoter of *alkB* gene; STOP, stop codons; RBS, ribosome binding site. Sites in parentheses were destroyed during cloning. Sequences of *alkB* and *luxA* open reading frames are in capital letters.

DNA manipulations. Plasmid DNA isolations, ligations, and transformations were carried out according to well-established procedures (26). Restriction enzymes and other DNA-modifying enzymes were obtained from Amersham International plc.; Boehringer GmbH (Mannheim, Germany); Gibco BRL, Life Technologies, Inc.; and Pharmacia (Uppsala, Sweden). DNA fragments were isolated from agarose gels with the Geneclean kit (Bio 101, Inc., LaJolla, Calif.) or QIAquick spin columns (Qiagen GmbH, Hilden, Germany).

Medium and growth conditions. *E. coli* strains were grown in Luria broth (LB) medium. Cell growth was monitored by measurements of the optical density at 600 nm in a Uvikon 800 spectrophotometer (Kontron Instruments AG, Basel, Switzerland). The following antibiotics were added to the medium when required: tetracycline (Tc) at 10 μg/liter and ampicillin (Ap) at 100 μg/liter. Strains were routinely grown at 37°C unless specified otherwise.

Preparation of the inoculum for induction experiments. The finally constructed strain, *E. coli* DH5α(pGEc74, pJAMA7), was used for induction experiments and for the quantification of linear alkanes. The inoculum for induction experiments was prepared as follows. Two milliliters of an overnight culture of *E. coli* DH5α(pGEc74, pJAMA7) was transferred into 100 ml of LB (Tc Ap) medium. The cells were incubated at 30°C for about 6 h until an optical density at 600 nm of 0.55 was reached. Afterwards, the culture was immediately put on ice for 15 min, and 20 ml of ice-cold sterile glycerol (87% [vol/vol]) was added. The mixture was kept on ice while it was divided into 650-μl portions in 1.5-ml Eppendorf tubes, which were then frozen in liquid nitrogen for 3 s and stored at −80°C.

Induction experiments with the whole-cell biosensor *E. coli* DH5α(pGEc74, pJAMA7). Induction experiments were carried out in 10-ml glass tubes that were tightly closed with glass stoppers to avoid the evaporation of volatile compounds. Each assay contained 5.7 ml of antibiotic-free LB medium, 0.3 or 0.6 ml of biosensor cells, and 60 μl of a stock solution of a potential inducer compound dissolved in ethanol. Assay concentrations of octane were between 24 nM and 6.3 μM. Other compounds were added at concentrations of 5 μM. The negative control contained 60 μl of ethanol. Positive controls were amended with 60 μl of freshly prepared octane stock solution (in ethanol) of the desired concentration, usually 11.2 μg/liter (98 nM).

The assay was carried out as follows. Frozen stocks of *E. coli* DH5α(pGEc74, pJAMA7) biosensor cells were thawed in a water bath at 25°C for 2 min. The biosensor cells were then placed back on ice until immediately prior to the inoculation of the assay. Induction experiments were started by the addition of the biosensor cells. During induction, the glass tubes with the assay mixtures were incubated at 30°C in a rotary shaker at 200 rpm. Samples of 200 μl were taken every 15 min during a period of 75 min to determine the amount of light emitted. If not stated otherwise, the sample taken after 1 h was used for comparison of inducer activities and for octane measurements.

Measurement of the light emission. Two hundred-microliter samples of assay mixtures were placed in a Microlite 1 microtiter plate (Dynatech Industries, Inc., McLean, Va.). Light emission was measured at 30°C in a Microlumat LB960 luminometer (Berthold AG, Regensdorf, Switzerland). Measurement was started after the automated injection of 25 μl of a decanal stock solution into the reaction assay. Along with the decanal injection, air was dispersed into the assay in order to supply the luciferase reaction with oxygen. The decanal stock solutions were prepared in a 1:1 mixture of water and ethanol. To optimize the decanal concentration in the assay, decanal was added to the reaction assay at final concentrations of between 0 and 5 mM. After the addition of the decanal substrate, kinetic light emission was monitored for 60 s. Subsequent routine measurements were done at a final decanal concentration of 2 mM. Light output was integrated from 20 to 30 s after the decanal injection.

Groundwater samples. Groundwater samples were collected from a diesel oil-contaminated site. Except for sample S6, all samples were taken after 40 to 300 liters of groundwater had been pumped from the respective wells. Sample flasks (0.1 or 1 liter) were completely filled with groundwater, without headspace, and stored at 4°C until further treatment. Sample S6 was collected from the bore hole without prior pumping of water. This sample was covered with a visible oil film.

Detection of short-chain-length alkanes in contaminated groundwater with *E. coli* DH5α(pGEc74, pJAMA7). Groundwater samples were assayed for linear alkanes in glass tubes containing 4.7 ml of groundwater, 1 ml of sixfold concentrated LB, and 0.3 ml of biosensor cells. The blank and the positive control contained nanopure water instead of groundwater samples, and the positive control also contained 11.2 μg of octane per liter (98 nM). The assay and the measurement were then carried out according to the standard procedure as described above. In order to quantify inhibitory effects on luciferase activity, each groundwater sample was spiked with 11.2 μg (98 nmol) of octane per liter, and light emission was compared to that for a positive control containing the same concentration of octane in water.

Chemical analysis. For chemical analysis, the groundwater samples were extracted with distilled pentane that had been spiked with 1.15 μM Cl-octane as the internal standard. Samples were concentrated 30-fold upon extraction. Two microliters was injected into a gas chromatographic mass spectrometer (GC-MS) (model MD800; Fisons Instruments, Manchester, United Kingdom) equipped with a 30-m DB-XLB (inner diameter, 0.25 mm; film width, 0.25 μm) column (J & W Scientific, Folsom, Calif.). The operating conditions were as follows. The GC was programmed for a temperature range from 313 to 498 K at a heating rate of 10 K/min. The MS detector was operated in the single ion mode recording ions with *m/z* values of 43 ± 0.2 (mean ± standard deviation), 57 ± 0.2, 71 ± 0.2, and 85 ± 0.2 typical for alkanes and 69 ± 0.2, 91 ± 0.2, 93 ± 0.2, and 105 ± 0.2 for the Cl-octane standard. Linear alkanes were identified by retention time and by their mass spectra. Aromatic compounds were identified by MS analysis.

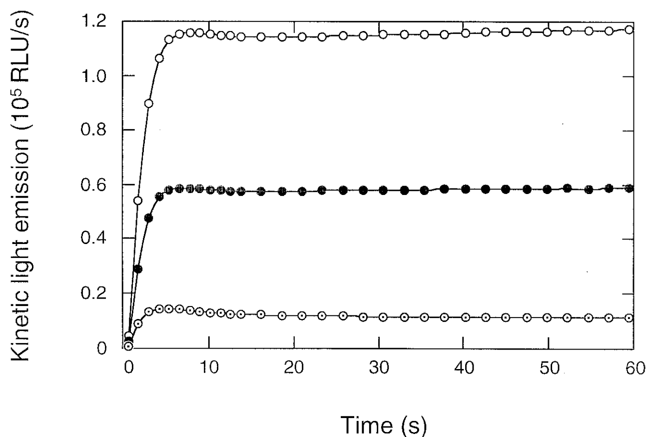

FIG. 2. Light emission kinetics immediately after injection of 2 mM decanal into octane-induced whole-cell biosensor *E. coli* DH5α(pGEc74, pJAMA7). Octane concentrations were 0.288 μM (●) and 1.44 μM (○). ⊙, control (no octane).

Chemicals. Agarose was obtained from Gibco BRL, Life Technologies, Inc. Yeast extract and tryptic casein were purchased from Biolife S. r. l. (Milan, Italy). Sodium chloride and glycerol (analytical grade) were obtained from Fluka Chemie AG (Buchs, Switzerland). Antibiotics were also purchased from Fluka. Decanal was purchased from Sigma Chemical Co. (St. Louis, Mo.). All inducer substances and solvents (analytical grade) were purchased from Fluka. Diethylether was further purified by distillation over sodium and subsequent filtration through Alox (I).

Data analysis. Quantification of alkane concentrations was done on experiments which were performed at least three times. Student's *t* test analysis at the 0.05 level was performed to check results for significance. Curve fits were done by linear or nonlinear least-squares analysis.

RESULTS

Optimization of the decanal substrate concentration for the luciferase activity of *E. coli* DH5α(pGEc74, pJAMA7). The luciferase activity in the *E. coli* recombinant strain carrying the cloned *alkS* regulator gene (on pGEc74) and the *alkB* promoter region of *P. oleovorans* fused to the *luxAB* genes of *V.*

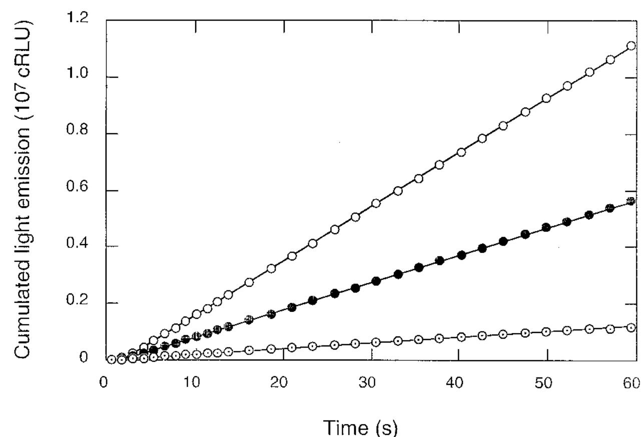

FIG. 3. Cumulated light emission after injection of 2 mM decanal into an induced culture of *E. coli* DH5α(pGEc74, pJAMA7). Symbols: ⊙, no octane; ●, 0.288 μM octane; ○, 1.44 μM octane. cRLU, cumulated RLU.

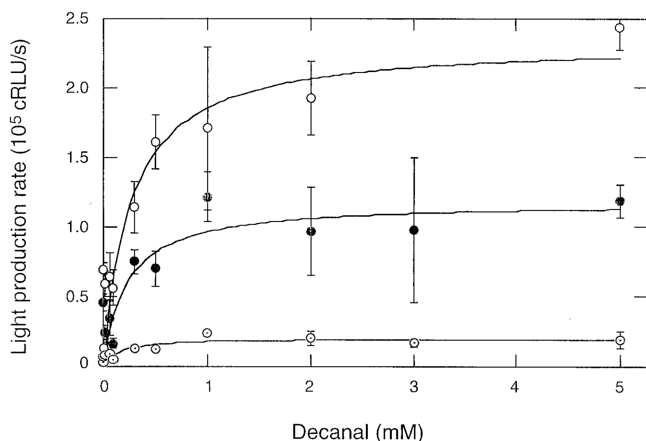

FIG. 4. Light production rate as a function of decanal concentration. Octane concentrations used to induce *E. coli* DH5α(pGEc74, pJAMA7) were no octane, ⊙; 0.288 μM, ●; and 1.44 μM, ○. The induction period was 60 min.

harveyi (pJAMA7) was readily inducible with octane, a typical inducer of the *alk* genes in *P. oleovorans*, indicating that both *alkS* and *alkB'-luxAB* transcription were functioning as expected (Fig. 2). No induction of luciferase expression by octane was observed in *E. coli* carrying only plasmid pJAMA7 (results not shown), indicating that this induction required the presence of AlkS.

One important prerequisite for reliable alkane measurements is the saturation of the cellular luciferase with decanal. The kinetic response of the luciferase showed no lag after the addition of decanal. The light emission reached a maximum after about 5 s and then remained almost stable. The cumulative light emission, calculated as the product of the luciferase reaction sequence, increased linearly with time, except during the first 5 s; this may have been due to the transport processes needed to achieve steady-state reaction rates (Fig. 3).

Light production rates (i.e., the slopes of the linear increase in the cumulative light production) showed a dependency on the decanal concentration that could be fitted reasonably well by Michaelis-Menten kinetics (Fig. 4). Saturation of the luciferase activity was observed for decanal concentrations above 2 mM for all octane concentrations tested. The half-saturation constants (K_m) were 0.2 mM for cultures induced with octane and 0.1 mM for cultures incubated without octane. V_{max} values depended on the octane concentrations and probably reflected the amount of luciferase enzyme present in the cells after induction. For practical reasons related to decanal solubility, we chose to use a decanal assay concentration of 2 mM (stock concentration of 18 mM in ethanol-water) in all further experiments.

Time-dependent induction of whole-cell biosensors *E. coli* DH5α(pGEc74, pJAMA7) and calibration with octane. An increase in light emission occurred during the first 80 min of induction of the biosensor with octane (Fig. 5). The optical density at 600 nm after 80 min increased slightly from 0.06 to about 0.08. After about 60 to 80 min, the total light output levelled off. The background activity remained constant at about 3×10^5 relative light units (RLU) during the whole experiment. With water-saturated octane concentrations (720 μg/liter; 6.3 μM), a maximum 20-fold increase for the luciferase activity compared to that for a control measurement without octane was observed after about 80 min of incubation.

Light output was calibrated against octane concentration at different induction periods shorter than 75 min. Light emission showed a saturation-type dependency on octane concentration, at any induction time (Fig. 6). Data points could be fitted with a hyperbolic equation, with good correlation for induction times over 15 min ($r > 0.95$). At low octane concentrations, a linear correlation also fitted the data. The linear range was extended most for an induction time of 60 min and reached from 2.8 to 90 μg of octane per liter (24.5 to 790 nM) (Fig. 6). At this induction time, background light emission without octane was 2.7×10^5 RLU. The lowest octane concentration tested (2.8 μg/liter; 24.5 nM) resulted in a 1.4-fold increase of light emission after 1 h of induction. This increase was significant at the 5% level ($n = 5$, $P < 0.05$). For 90 μg/liter (790 nM), light emission was 8.4 times background light emission.

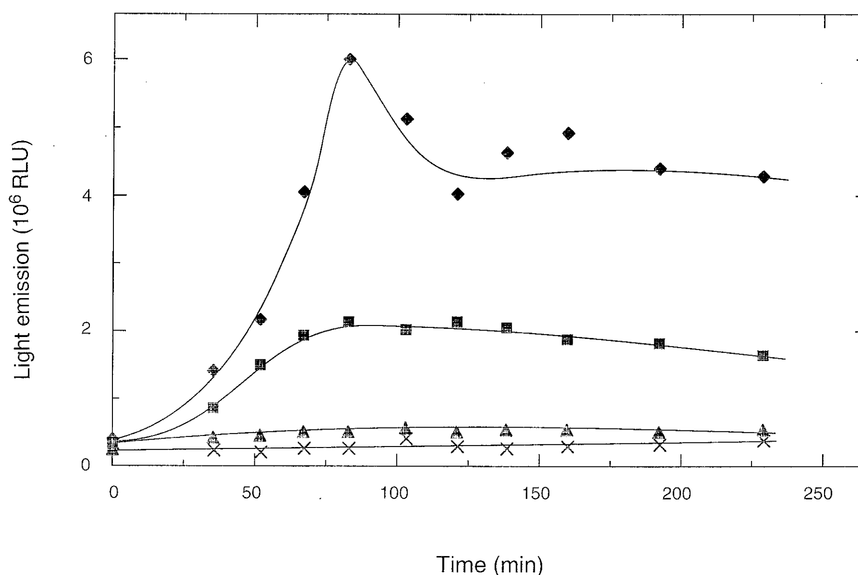

FIG. 5. Time-dependent light emission of *E. coli* DH5α(pGEc74, pJAMA7) after induction with different octane concentrations. Symbols: ◆, 6.3 μM octane; ■, 0.63 μM octane; ▲, 63 nM octane; ×, no octane.

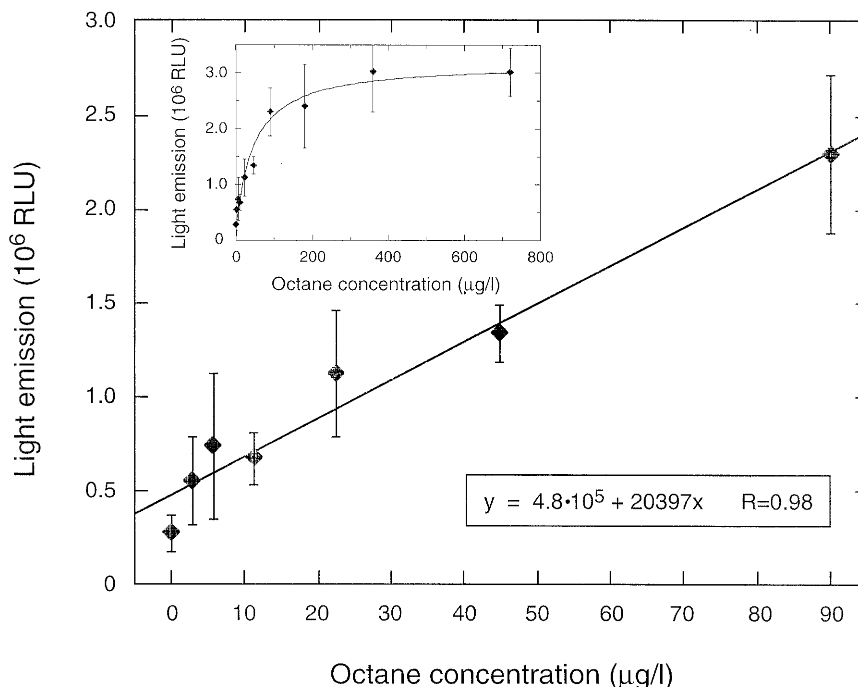

FIG. 6. Light emission by *E. coli* DH5α(pGEc74, pJAMA7) after a 60-min induction period as a function of octane concentration.

Induction and inhibition of the luciferase activity in *E. coli* DH5α(pGEc74, pJAMA7) with compounds other than octane. A range of other alkanes, alkane mixtures, and several other compounds (at concentrations of 5 µM) was tested for induction of the biosensor (Table 2). Significant induction was found for the alkanes from pentane to decane, whereas long-chain-length linear alkanes such as dodecane and hexadecane did not induce luciferase activity in the biosensor. High-boiling petroleum ether, a relatively undefined alkane mixture, led to an induction that was 16% of that with octane. A relatively strong induction was observed for the singly branched alkane 3-methylheptane (36%). No induction occurred with the gratuitous

TABLE 2. Relative luciferase activity after induction with different compounds

Compound[a]	Relative induction[b]	Compound	Relative induction
Linear alkanes		Alicyclic hydrocarbons	
Pentane	**13**[c] (ND)[d,e]	Cyclohexane	9 ≤2
Hexane	**44** (10)	Methylcyclohexane	11 ≤2
Heptane	**81** (85)	Dimethylcyclohexane	11 (ND)
Octane	**100** (100)	Cycloheptane	11 (ND)
Nonane	**100** (90)		
Decane	**69** (36)	Aromatic hydrocarbons	
Undecane	6 (32)	Benzene	10 ≤2
Dodecane	11 (2–3)	Toluene	10 ≤2
Hexadecane	11 (ND)	*m*-Xylene	11 ≤2
		Trichlorobenzene	9 (ND)
Petroleum ether[f]			
Low boiling (30–45°C)	10 (ND)	Alkylbenzene, Hexylbenzene	12 ≤2
High boiling (50–70°C)	**16** (ND)		
		Polycyclic aromatic hydrocarbons	
Branched alkanes		Naphthalene	10 (ND)
Heptamethylnonane	11 (ND)	1-Methylnaphthalene	8 (ND)
3-Methylheptane	**36** (88)	DCPK	9 (86)[g]
Pristane	11 (ND)	Background	8[h] (≈2)

[a] Assay concentration of individual compounds was 5 µM. Compounds were added to the assay as ethanol stock solutions.
[b] Induction was measured after 69 min. Light output (expressed in percent) was related to octane-induced light emission, arbitrarily set at 100%.
[c] Bold-faced numbers indicate significant induction at the 5% level ($5 < n_1 + n_2 < 8$, $P < 0.05$, unpaired, one-tailed test).
[d] ND, not determined.
[e] Values in parentheses refers to the data obtained by Wubbolts (37) with a P_{alkB}-*cat* reporter.
[f] Petroleum ether refers to commercially available alkane mixtures. These mixtures were added in the same amount as pentane (low-boiling petroleum ether) and hexane (high-boiling petroleum ether), respectively.
[g] The assay concentration of DCPK was 0.05% (440 µM).
[h] Background light emission was determined by adding the same amount of pure ethanol instead of inducer solution to an assay.

TABLE 3. Inhibition of the octane-induced light emission in *E. coli* DH5α(pGEc74, pJAMA7) by different groups of related compounds

Substance class	Compounds[a]	Inhibition (%)[b]
Linear alkanes	Pentane, hexane, heptane, decane, dodecane	6
Alkane mixtures	Petroleum ether (low and high boiling)	11
Branched alkanes[c]	Heptamethylnonane, 3-methylheptane, pristane	0
Alicyclic hydrocarbons	Cyclohexane, methylcyclohexane, cycloheptane	19
Aromatic hydrocarbons	Benzene, toluene, *m*-xylene	12
PAHs[c]	1-Methylnaphthalene	0
Alkylbenzenes	Hexylbenzene	14
Biphenyls	2-Hydroxybiphenyl[d]	85
Other	DCPK	37

[a] Assay concentration for each individual compound was 5 μM.
[b] Inhibition refers to the percentage of inhibition of the luciferase activity in the presence of these compounds with octane compared to that of an assay with only octane present. Light response was measured after 69 min.
[c] PAHs, polycyclic aromatic hydrocarbons. Inhibition was measured after 54 min of incubation.
[d] 2-Hydroxybiphenyl was added to the assay in crystalline form.

inducer dicyclopropylketone (DCPK) (37) at a concentration of 5 μM, but the bioreporter was induced at the previously used 440 μM (0.05%) (results not shown).

Several of the compounds that were tested for their abilities to induce luciferase activity in *E. coli* DH5α(pGEc74, pJAMA7) were then mixed as groups of substance classes and added to induction assays together with octane in order to determine their influence on the biosensor response to octane (Table 3). Since octane was present at a concentration (5 μM) that was high enough to induce maximal light emission (Fig. 3), one would expect the mixtures to exhibit a reducing effect, if any, on the induction. This effect was indeed observed (Table 3), most strikingly for 2-hydroxybiphenyl, which led to an 84% decrease in luciferase activity.

Application of the whole-cell biosensor in contaminated groundwater. We used the *E. coli* biosensor to measure the alkane concentrations available to the cells in samples from a heating oil-contaminated groundwater site. In this area, 30 m^3 of diesel oil had reached the groundwater table, probably in 1991, due to an undetected leak in a pipeline. A small but detectable induction of the luciferase activity of *E. coli* DH5α(pGEc74, pJAMA7) was observed in most of the samples taken from eight wells (Table 4). Since we could not differentiate between possible inducers, the data were expressed as octane equivalents. Compared to a negative control without any additional alkanes or groundwater present, the induction was significant except for samples P7 and P9 ($n_1 = n_2 = 3$, $P < 0.05$). Possible inhibitory effects were assessed by spiking all samples with a known amount of octane (98 nM) and measuring the reduction in the total light output compared to that for a positive control containing the same octane concentration in deionized water. In all samples, a significant average reduction of 23% of the light emission was observed ($4 < n < 6$, $P < 0.05$) (Table 4).

We also analyzed the samples by GC-MS in order to verify the outcome of the induction experiments (Table 4; Fig. 7). In the oil film floating on sample S6, alkanes with chain lengths above C_8, among them the inducer compounds C_9 to C_{11}, were

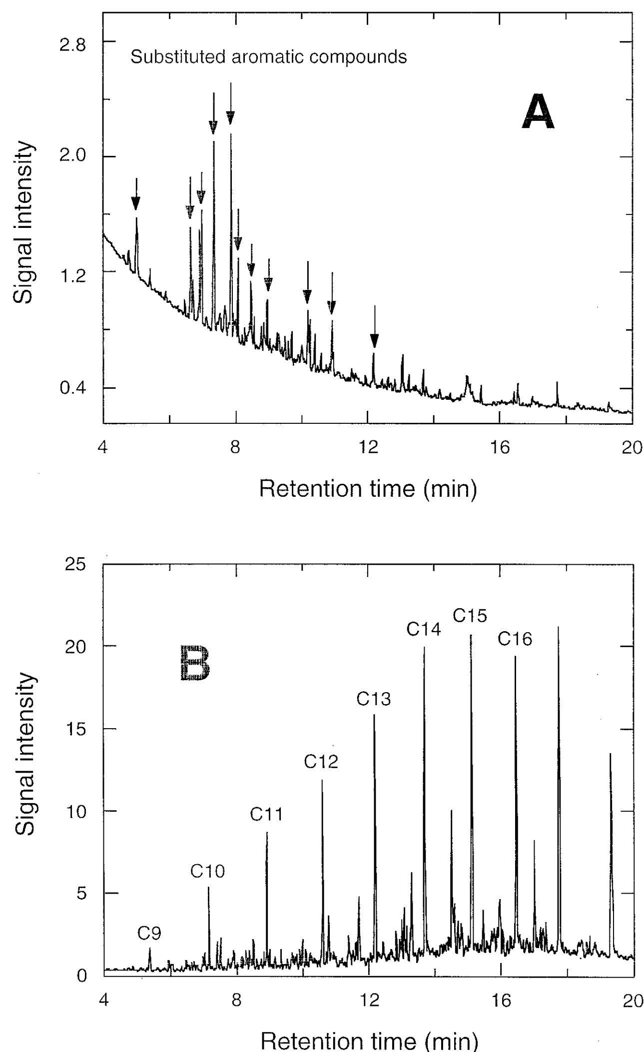

FIG. 7. GC-MS total ion chromatogram of groundwater from sample S6. (A) Water without oil film. Arrows indicate alkyl-substituted aromatic compounds by GC-MS analysis. (B) Oil-water emulsion. Linear alkanes are indicated by number of carbon atoms.

TABLE 4. Analysis with *E. coli* DH5α(pGEc74, pJAMA7) of groundwater at diesel oil-contaminated site

Sample	Inhibition (%)[a]	Octane equivalents (nM)	
		Uncorrected[b]	Corrected[c]
P1	20 (±9)	3.04 (±0.52)	3.79 (±1.00)
P4	14 (±9)	3.23 (±0.61)	3.77 (±1.03)
P5	18 (±11)	2.94 (±1.31)	3.61 (±1.84)
P7	25 (±14)	<0	<0
P9	27 (±15)	0.52 (±1.55)	0.71 (±1.73)
PS9	36 (±12)	1.82 (±0.97)	2.82 (±1.61)
S6	25 (±8)	14.35 (±4.28)	19.10 (±6.61)
S7	15 (±9)	2.61 (±0.68)	3.08 (±1.04)

[a] Inhibitory effect of the groundwater samples on the biosensor performance was measured by spiking samples with 98 nM octane and determining the reduction of the total light output. Results are means (± standard deviations).
[b] Values were calculated by extrapolating standard light output data (Fig. 6) to very low octane concentrations by using a hyperbolic function. Detection limits for *n*-alkanes in the chemical analytical procedure were 15 nM for octane, 5 nM for nonane, 20 nM for decane, and 20 nM for undecane.
[c] Corrected for the inhibitory effect of the sample on the biosensor performance.

clearly identified (Fig. 7B). When the oil emulsion was carefully removed in a separatory funnel, the resulting water fraction contained practically no alkanes. The dominant peaks in sample S6 were identified by MS as alkyl-substituted aromatic compounds (Fig. 7A). In all aqueous samples, the concentrations of linear alkanes were below the limit of detection of the applied GC-MS method (between 5 and 50 nM, depending on the measured compound). Middle-chain-length alkanes (C_8 to C_{11}) were present at concentrations below 20 nM each, and longer-chain-length alkanes (C_{12} to C_{16}) were present at concentrations below 50 nM each.

DISCUSSION

We described the construction, characterization, and application of a bioluminescent whole-cell biosensor for the measurement of water-dissolved concentrations of linear alkanes. The basis of our biosensor system was *E. coli*, containing the gene for the sensor protein AlkS, and a transcriptional fusion between P_{alkB} and the promoter-free *luxAB* genes on two different compatible plasmids. *E. coli* DH5α(pGEc74, pJAMA7) showed a specific response and a good sensitivity for middle-chain-length linear alkanes and one branched alkane.

The biosensor was capable of sensing and reporting octane concentrations as low as 24.5 nM. This concentration corresponded to the presence of about 20 molecules of octane in the cytoplasm of each cell, assuming a cell volume of 1.6 μm^3 and an equal distribution of octane between the bulk liquid and the cytoplasm. In a similar calculation, DiMarco and coworkers observed that roughly 50 molecules of *p*-hydroxybenzoate per cell were sufficient to lead to an activation response of the transcriptional activator PobR (7). Other transcriptional regulators for catabolic gene expression typically responded to inducer concentrations below a few micromoles per liter (6, 7, 16, 28, 29). Transcriptional regulators often exhibit relaxed specificities towards compounds that are structurally more or less related (28, 29, 37). The AlkS-*alkB′*-*luxAB* system in *E. coli* DH5α showed a specific induction for short- and middle-chain-length alkanes. These results were mostly in agreement with those from a previous study (37) with *E. coli* W3110(pGEc289, pGEc74). This strain harbored a P_{alkB}-*cat* (chloramphenicol acetyl transferase) transcriptional fusion.

The use of *E. coli* as the host strain for the alkane-sensing and alkane-regulated luciferase response has all the advantages of working with *E. coli*. The host strain apparently expressed *alkS* and recognized the *alkB* promoter well enough to allow a workable system. However, a relatively high background expression from P_{alkB} occurred under uninduced conditions, even though the expression of *alkB′*-*luxAB* was transcriptionally shielded. Perhaps this was due to an incomplete repression or to the presence of weak *E. coli* promoters in this part of the *alkB* sequence. In addition, the current host strain carries the *alkB′*-*luxAB* fusion on a high-copy-number plasmid. Consequently, a weak basal level expression of *alkB′*-*luxAB* would lead to a high background luciferase activity. This high background activity may be further reduced by introducing the regulatory gene and the P_{alkB}-*luxAB* fusion on the same plasmid or on the chromosome of *E. coli.*

As expected on the basis of results from other studies (1), we observed that the light emission of *E. coli* DH5α(pGEc74, pJAMA7) was strongly dependent on the concentration of decanal in the assay. By testing different decanal concentrations, we determined that at concentrations of 2 mM and above, the available luciferase in the cells was saturated with substrate. Therefore, at those substrate concentrations, light emission is independent of decanal concentration and directly reflects the amount of luciferase enzyme. Although it is known that high decanal concentrations can inhibit the luciferase enzyme (18) and can be cytotoxic (1), we did not observe such effects in our assay. Interestingly, the typical time-kinetic response to higher decanal concentrations (Fig. 1) of light emission by the biosensor in our study differed from the responses observed in other studies (1).

Our biosensor strain carried only the regulatory elements of the *alk* operon and was consequently unable to metabolize linear alkanes. Such a biosensor is a good tool for the rapid, unequivocal measurement of specific pollutants in contaminated water or soil samples and shows the instantly available concentration of inducing compounds. For measuring substrate fluctuations rather than momentary concentrations, a biosensor which both senses and degrades a particular compound is favored. Such a biosensor system is the extensively studied naphthalene/salicylate-degrading sensor strain *Pseudomonas fluorescence* HK44 (4, 15, 16, 20). Besides the ability of the biosensor strain to degrade the inducing compound, turnover rates of cellular luciferase activity play an important role in such applications (15).

In contaminated sites, where hundreds of different chemicals besides the inducer compounds are present and may interact with a biosensor, it is clearly impossible to check the effect of these groups of compounds or of individual compounds, even if the compounds have been identified by chemical analysis. Possible inhibitory effects can be addressed by adding a known amount of optimal inducer (in this case, octane) to the unknown samples and measuring induction differences. In the groundwater samples tested, we determined that the biosensor underestimated the bioavailable alkane concentration by approximately 25%, probably due to inhibition. For routine measurements, some research groups (3) considered spot checking probably sufficient. An alternative strategy to account for effects of noninducing compounds on the biosensor performance is the use of isogenic biosensor strains that exhibit constitutive bioluminescence (16) or the introduction of a second, constitutively expressed luciferase that emits another wavelength (36). However, the use of such systems invokes other uncertainties, since the AlkS system might respond differently to the presence of noninducing compounds than to that of a constitutively expressed *luxAB* construct.

By applying our biosensor strain, we could monitor the presence of a small inducible fraction of (most likely) middle-chain-length alkanes in heating oil-contaminated groundwater samples. Due to the specificities of the sensor's response, the possibility that some related compounds added to the inductive effect could not be excluded. In most samples, no individual alkanes could be detected by chemical analyses. Indeed, the octane-equivalent concentrations detected with the biosensor were below the detection limits for most alkanes reached by GC-MS (5 to 50 nM). Further improvements are needed to verify the biosensor measurements for alkanes in this low-concentration range.

ACKNOWLEDGMENTS

P.S. and M.C.M.J. contributed equally to this paper and should therefore both be considered as first authors.

We thank M. Wubbolts (Institute for Biotechnology, ETH Zürich, Switzerland) for kindly providing plasmids pGEc47 and pGEc74 and J. Kuhn (Israel Institute of Technology) for plasmid pHG171-luxAB. Also, we thank P. Höhener and J. Zeyer (Institute for Terrestrial Ecology, ETH Zürich, Switzerland) for giving us the opportunity to collaborate and participate in investigating a groundwater spill.

This work was supported by the Swiss Priority Program Environment.

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