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Bioluminescent Reporter Bacteria Detect Contaminants in Soil Samples

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ABSTRACT

Reporter strains of bacteria were tested using soil samples from several sites near a leaking fuel oil storage facility. The reporter bacteria utilized the bioluminescent *lux* genes from *Vibrio fischeri*, which were transcriptionally fused to catabolic gene sequences. The catabolic genes of interest specified the degradation of toluene (from the TOL plasmid) and naphthalene (from the NAH7 plasmid and from a NAH plasmid recently isolated). The results indicated that two soil samples were contaminated with both toluene (or xylene) and naphthalene. These data were useful in describing the extent of contamination at the site.

Index Entries: Naphthalene; toluene; bioluminescent; bioreporter; bioavailability.

INTRODUCTION

Bioremediation has become an increasingly useful means of handling toxic and hazardous waste spills, both in soil and water. There are several reasons for this phenomenon. Bioremediation often results in the complete destruction of contaminants, or at least to concentrations within regulatory guidelines. It is frequently more cost-effective than alternative technologies, such as incineration. It is sometimes the only practical alternative for certain remediation projects, such as contaminated aquifers or large hazardous waste plumes within subsurface soils.

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Many bioremediation projects have already been described, and many more are currently in operation (*1*). Most of these projects are performed without characterization of the microbial community that is degrading the contaminants, or of the conditions that favor catabolism. In order to address this problem, we have created bioreporter strains that produce an assayable signal whenever the genes for catabolism are expressed. Many bioreporter genes could have been chosen, although the versatility of the *lux* gene system makes it an attractive alternative.

The *lux* genes produce visible light in their host bacterium, *Vibro fischeri* (*2*); these genes have been cloned onto convenient plasmid vectors (*3*). We have fused promoters from catabolic operons to these genes, effectively placing expression of bioluminescence under the control of the catabolic genes. For this system to function, the appropriate regulatory gene(s) must be present as well. In the presence of the inducer contaminant the catabolic genes will be induced, and the strain will produce visible light that can be detected and quantified.

In assessing the potential of a site for bioremediation work, it is important to determine not only the presence and identity of the chemical contaminants, but also whether those contaminants are bioavailable to the catabolizing bacterial population. This report describes the use of bioluminescent reporter strains of bacteria for the evaluation of the presence and bioavailability of specific contaminants in soil and water samples. We demonstrate that detection of compounds is sensitive, even in complex soil samples.

METHODS

Strains and Plasmids

The *Pseudomonas fluorescens* HK44 strain catabolizes naphthalene and salicylate. It contains a *nah-lux* fusion, and has previously been described as a bioreporter for environmental samples (*4*). *Pseudomonas putida* RB1351 is also a naphthalene and salicylate degrader. It contains a different *nah-lux* construction, and has also been described (*5*). The *Pseudomonas putida* RB1401 strain degrades toluene and xylenes, and contains a *xyl-lux* fusion plasmid (*6*). This strain was constructed from *P. putida* mt-2 and contains the archetypical TOL plasmid. Strains were maintained and stored using standard methods (*7*). Important details of these *lux* fusions are shown in Fig. 1.

Media and Reagents

LB medium has been described (*7*). YEPG medium contains (in g/L) yeast extract (0.2), polypeptone (2.0), glucose (1.0), and ammonium nitrate (0.2). After autoclaving, 100 mL of a sterile 0.5*M* phosphate buffer was added. Cells were grown in minimal medium for all assays. The formula

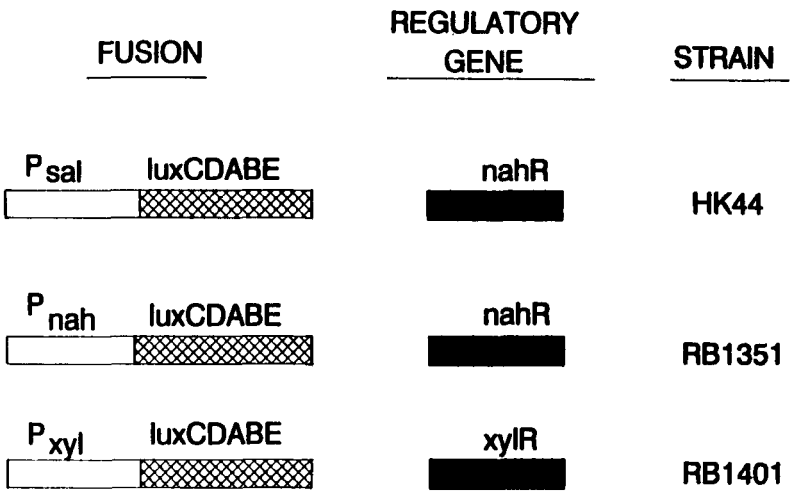

Fig. 1. Bioreporter fusions. The construction of the three strains is shown. The regulatory gene is present on a separate plasmid in each strain. Psal denotes the promoter of the lower pathway of naphthalene catabolism, whereas Pnah denotes the upper pathway. Pxyl is the promoter from the upper pathway of toluene catabolism.

Table 1
Composition of Minimal Medium

Minimal medium (all figures are per liter)	
$MgSO_4 \cdot 7H_2O$	0.1 g
NH_4NO_3	0.2 g
0.5*M* Phosphate buffer	100 mL
Trace elements	0.1 mL
Glucose	1.0 g
Trace elements (all figures are per liter)	
MgO	10 g
$CaCl_2$	2.94 g
$FeCl_3 \cdot 6H_2O$	5.4 g
$ZnSO_4 \cdot 7H_2O$	1.44 g
$CuSO_4$	0.25 g
H_3BO_4	62 mg
$Na_2MoO_4 \cdot H_2O$	0.49 g

is presented in Table 1. Selection for the recombinant plasmid was with tetracycline (15 mg/L) for HK44 and kanamycin (50 mg/L) for RB1351 and RB1401. Toluene was obtained from Aldrich Chemical (Milwaukee, WI). Naphthalene was obtained from Sigma Chemical (St. Louis, MO). All other listed antibiotics, chemicals and reagents were obtained from Sigma.

Soil and water samples were obtained as part of a field-scale investigation of petroleum contamination on Kwajalein Island, a US Army installation in the Republic of Marshall Islands. They were transported on ice in EPA vials and stored at 4°C until assayed.

Light Measurement

An Oriel (Stratford, CT) digital display model 7070 with a photomultiplier tube model 77340 connected to a flexible liquid-light pipe and collimating beam probe was used for all light measurements. The photomultiplier tube was sensitive to the region of the spectrum emitted by the bioluminescent bacteria (about 490 nm). Light was measured as an induced current, and so is reported as nanoamperes of induced current.

Assay Conditions

Bacterial cultures were taken from frozen aliquots and grown overnight in YEPG medium at 25°C. A 1:100 dilution was made into minimal medium and allowed to grow overnight. This culture was diluted 1:10 in minimal medium and grown with shaking (150 rpm) at 25°C for 3–4 h. After this time the optical density (546 nm) was in the range 0.35–0.40. Aliquots of 30 mL of these cells were concentrated by centrifugation (10 min at 8000*g*) and resuspended in minimal medium without glucose.

Because the contaminants under investigation, toluene and naphthalene, are volatile, samples were taken from the stock vials and used immediately. These samples, 2 g of soil or 2 mL of water, were mixed with 2 mL of the bacterial suspension described above and sealed in an EPA vial with a teflon lid. Assay vials were incubated at 27°C with constant shaking at 150 rpm. At time invervals the vials were inserted into a light-tight black box to which the flexible liquid-light pipe was attached, and bioluminescence was measured. Positive and negative controls were used with each test. Positive controls were performed with 10 mg naphthalene or 0.015 m*M* toluene added to the EPA vials before sealing. Duplicates of each test and control sample were used.

RESULTS

Naphthalene Detection

Both the HK44 and RB1351 strains were used with the samples for detection of naphthalene. The protocol was the same for each strain.

Test pits were dug on Kwajalein Island in five locations near the fuel tanks that were suspected of leaking. These were named TP01 through TP05. The numerical suffix for each sample indicates the depth, in inches, that the sample was obtained from at that site. In addition, water samples from the water table were examined.

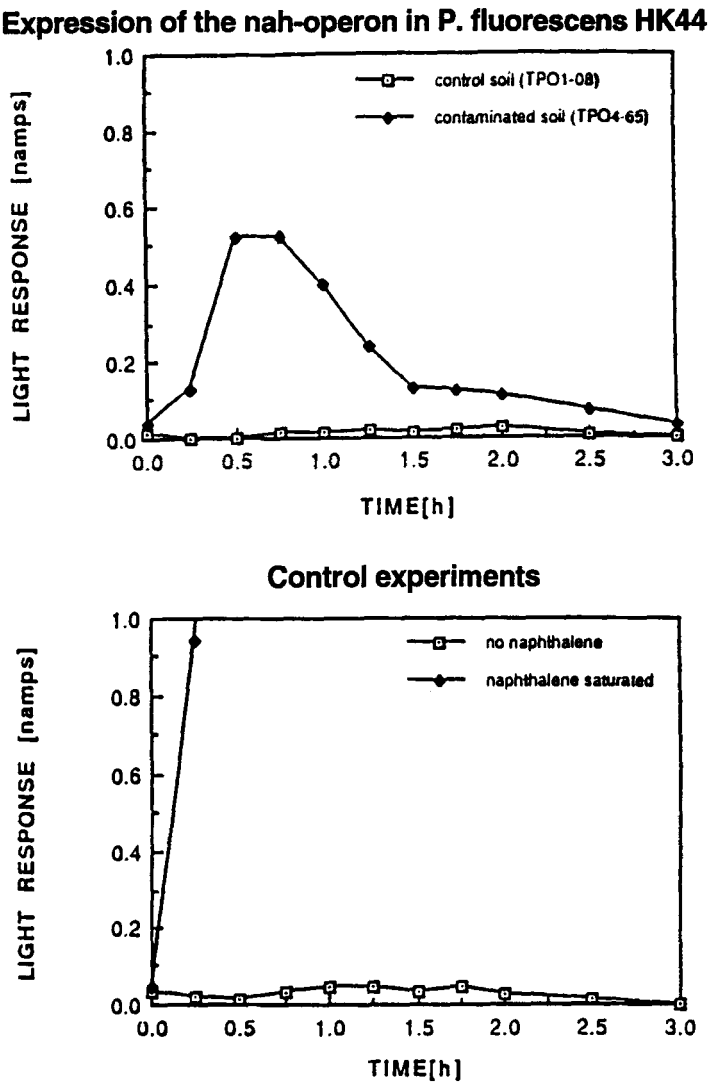

Fig. 2. Bioluminescence from the HK44 strain in response to incubation with test samples.

Figure 2 shows typical results with these samples. The negative control (Fig. 2, bottom) does not produce any appreciable bioluminescence throughout the 3-h time-course of the assay. The positive control (with 10 mg naphthalene added) shows a strong and rapid bioluminescent response, which reached a peak greater than 10 nanoamps and which continued at this high level throughout the timecourse of the experiment. Naphthalene has low solubility in water, and this amount is essentially a saturating amount. Light production will remain strong as long as naphthalene is present and no other nutrient becomes limiting (*8*).

An assay of the samples from the test pits clearly distinguished contaminated from uncontaminated samples (Fig. 2, top). An uncontaminated sample, such as TP01-08, produced no bioluminescence over the very low background amount. Contaminated samples, such as TP04-65, produced noticeable increases in bioluminescence after 1 h, and usually produced a maximum response within 2 h.

Assays using the RB1351 strain gave similar results to those described above (data not shown). The strains HK44 and RB1351 use different, though related, naphthalene degradation pathways. The major difference that should be noted for this work is that RB1351 utilizes a *nah-lux* fusion with the upper pathway of degradation, while HK44 utilizes a fusion with the lower pathway (Fig. 1). For the detection of naphthalene in environmental samples, these two fusions can be regarded as equally useful.

Toluene and Xylene Detection

The same samples were tested for the presence of toluene and ortho, meta, and paraxylene using the RB1401 strain. As shown in Fig. 3, the positive and negative controls clearly show the difference between induced and uninduced strains. The positive control contained toluene at 15 μM final concentration (Fig. 3, bottom). This low concentration was necessary since toluene can be toxic to bacterial cells. Typical results with the test samples are also shown (Fig. 3, top). Here again, a clear distinction is evident between contaminated (TP04-65) and uncontaminated (TP01-08) samples. The time course for positive results to become evident is also similar to the results using the naphthalene bioreporters.

Here again, the bioluminescent response is transient because toluene is a carbon and energy source for this strain. This is also true for *m*-xylene and *p*-xylene, although not for *o*-xylene, which is a gratuitous inducer of the *xyl* operons (*9*). In an experiment to determine whether the presence of a gratuitous inducer would markedly affect the outcome of a bioluminescence assay, we induced a pure culture of RB1401 with *o*-xylene, *m*-xylene, and *p*-xylene (Fig. 4). The strain was grown in LB broth, induced with a contaminant at 1 mM final concentration, and the bioluminescence determined. The results show that induction with either compound will result in a strong bioluminescent response, although the *o*-xylene result gives a higher peak. A rapid decline follows this high peak (data not shown). It appears that some constituent of the medium quickly becomes limiting under these conditions and causes the strain to cease light production, even though LB is a rich medium. This suggests that samples with *o*-xylene will still give a detectable bioluminescent response with this assay, although more frequent timepoints might be required.

Outcome of the Site Samples

Twelve samples were tested from the five test pits, including one sample from the water table. The results from the tests with the RB1401

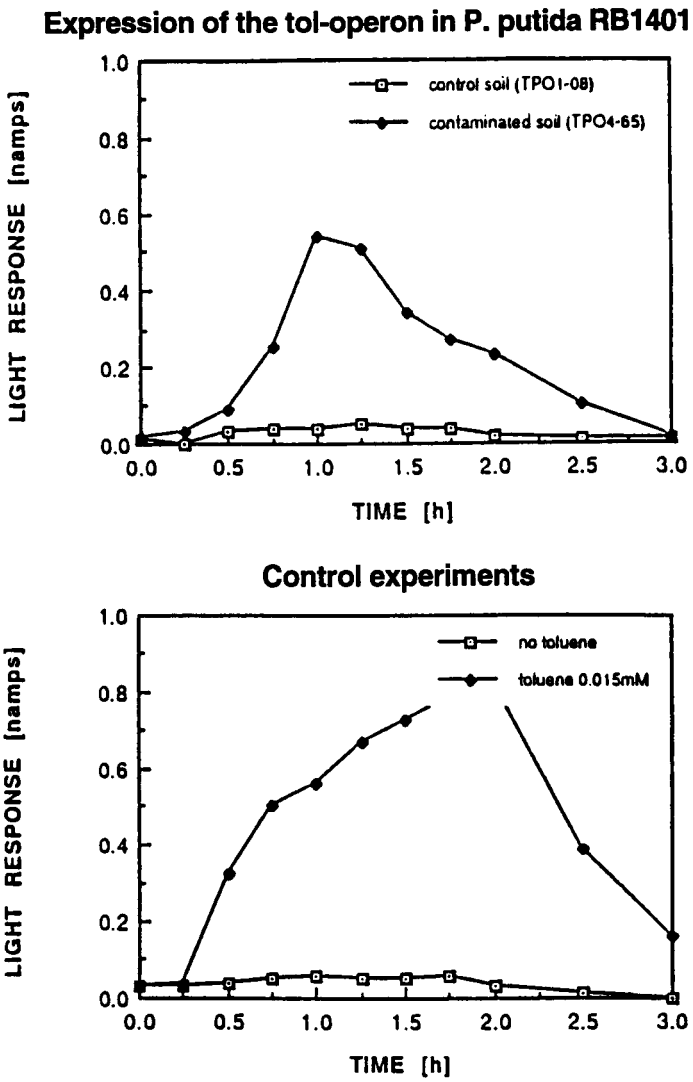

Fig. 3. Bioluminescence from the RB1401 strain in response to incubation with test samples.

(toluene, xylene) strain are presented in Fig. 5. Only two samples, TP04-65 and TP05-70, were found to contain detectable amounts of inducing contaminants. The same pattern was found for naphthalene presence using the HK44 strain. These two test pits were closest to the fuel tanks that had been suspected of leaking. Interestingly, sample TP05-62, which was only 8 in. above one of the contaminated sites, showed no evidence of contamination. In addition, the water sample from Test Pit 4, only about 8 in. below another contaminated sample, also showed no sign of contamination with this assay. The overall picture suggests a contamination problem that is localized to a relatively small area around the fuel tank.

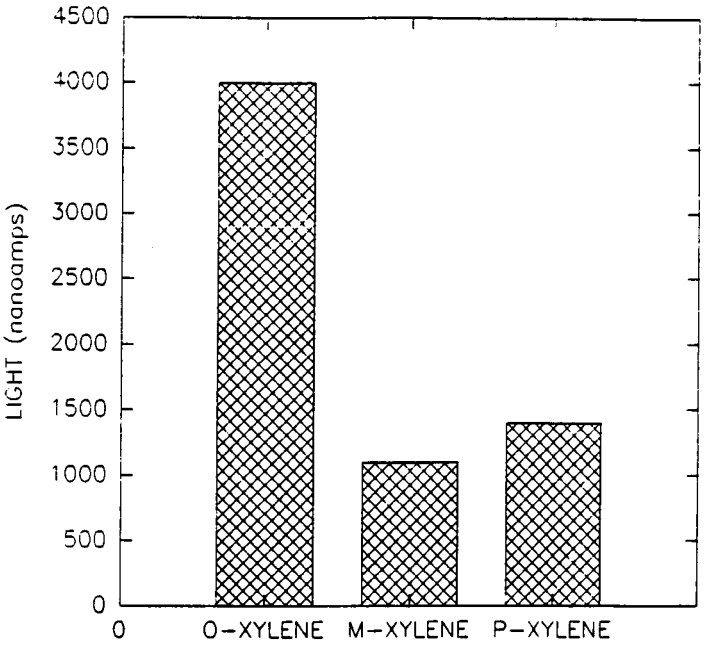

Fig. 4. Peak values of bioluminescence when strain RB1401 is induced with each of the xylenes (final concentration, 1 m*M*).

DISCUSSION

These results demonstrate that bioluminescent reporter strains can provide valuable data about the presence and bioavailability of specific contaminants in environmental samples. Using these results, it was possible to inform the sponsor where the regions of greatest contamination were located.

It is still possible to obtain false positive and false negative results with this assay, and we are continuing our research to determine how common these results would be. False positives might occur if some other inducer substrate, rather than a contaminant substrate, were present in the environmental sample. This could occur with the *nah-lux* bioreporter, for instance, if either salicylate or anthranilate were present. Both of these compounds are common in nature, although it is unlikely that they are ever found in abundance extracellularly, since they are good sources of carbon and energy for a variety of microorganisms. False negatives could result if significant quantities of metabolic inhibitors were present in the environmental samples. Inhibitors might be natural, such as antibiotic compounds, but are more likely to come from contaminated sites that contain compounds such as cyanides and chlorinated aromatics. False negatives can be discovered fairly easily, since adding the appropriate

Cross Section of Kwajalein Soils					
Light response using a tol-reporter					
Site Depth	TP01	TP02	TP03	TP04	TP05
[Inches]					
6		-			
8	-				
12			-		
32				-	
33	-				
56	-				
62					-
63		-			
65				+	
70					+
72			-		
W				-	

Fig. 5. A cross-section of the 12 samples from the 5 test pits (TP01-05). Symbols: (+) contamination with toluene or xylene; (−) no contamination detected; W = groundwater sample.

inducer compound to an identical sample should result in a bioluminescent response. The absence of such a response under these conditions would indicate that some other contaminant was adversely affecting the cells. Checking all negative samples using this method would be laborious, and spot checks are probably sufficient. The automation of this assay system might enable greater numbers of samples to be tested.

The usefulness of this assay is demonstrated clearly in the results of this study. Although many test pits were dug and many samples taken, only two closely associated samples showed inducing levels of contaminants. When a comparison of the volume of soil that was suspected of contamination is made with the volume of soil that was proven to be contaminated, it is clear that the actual contaminated area is a small fraction of the total. These data translate directly into treatment savings, since cost increases with the increase in soil that must be moved and treated. In effect, this assay pinpoints the areas that require remediation. In addition, this assay reveals whether the contaminants are bioavailable to the catabolizing bacteria. This is an important distinction, since other assays can report on the presence of specific compounds. Other assays, however, can not determine whether the contaminants are available to the microorganisms for catabolism.

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