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A Chromosomally Based *tod-luxCDABE* Whole-Cell Reporter for Benzene, Toluene, Ethybenzene, and Xylene (BTEX) Sensing

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A *tod-luxCDABE* fusion was constructed and introduced into the chromosome of *Pseudomonas putida* F1, yielding the strain TVA8. This strain was used to examine the induction of the *tod* operon when exposed to benzene, toluene, ethylbenzene, and xylene (BTEX) compounds and aqueous solutions of JP-4 jet fuel constituents. Since this system contained the complete *lux* cassette (*luxCDABE*), bacterial bioluminescence in response to putative chemical inducers of the *tod* operon was measured on-line in whole cells without added aldehyde substrate. There was an increasing response to toluene concentrations from 30 μg/liter to 50 mg/liter, which began to saturate at higher concentrations. The detection limit was 30 μg/liter. There was a significant light response to benzene, *m*- and *p*-xylenes, phenol, and water-soluble JP-4 jet fuel components, but there was no bioluminescence response upon exposure to *o*-xylene. The transposon insertion was stable and had no negative effect on cell growth.

Due to the widespread use of petroleum products and the current regulations requiring underground storage tanks to be upgraded, replaced, or closed by December 1998 (4), the number of petroleum-contaminated sites has abounded. Of particular concern for drinking water quality are the more water-soluble components, benzene, toluene, ethylbenzene, and xylenes (BTEX). Natural attenuation, which relies on in situ biodegradation of pollutants, has received a large amount of attention, especially for petroleum contaminants (15). While microorganisms capable of biodegradation of BTEX compounds are usually present at these sites, there is a need to know whether or not conditions are favorable for biodegradation to occur. A recent approach to determine whether compounds are bioavailable and what conditions are favorable for degradation is the use of whole-cell bioluminescent reporters (9).

Bioluminescent reporters have been widely used for the real time nondestructive monitoring of gene expression. Heitzer et al. (8) developed a quantitative assay for naphthalene bioavailability and biodegradation by using a *nah-lux* reporter strain constructed by King et al. (13) and expanded its use as an on-line optical biosensor for application in groundwater monitoring (10). Other *lux* fusions have been constructed for monitoring the expression of catabolic genes, including those for degradation of isopropylbenzene (21) and toluene (1, 5). *lux* fusions have also been constructed for monitoring heat shock gene expression (24, 25), oxidative stress, (3), the presence of Hg(II) (20), and alginate production (26). In all of these cases, the *lux* fusions were plasmid based and were constructed by placing the promoter of interest in front of the promoterless *lux* genes from *Vibrio fischeri* contained in pUCD615 (18).

In this study, a strategy was pursued to introduce a single copy of the *lux* fusion into the bacterial chromosome via a transposon delivery system. A mini-Tn*5* delivery vector constructed by Herrero et al. (11) provided the basic model for this work. By this approach, a *tod-lux* fusion was constructed and introduced into *Pseudomonas putida* F1 to examine the induction of the *tod* operon when exposed to BTEX compounds and aqueous solutions of JP-4 jet fuel constituents. Since this system contains the complete *lux* cassette (*luxCDABE*), bacterial bioluminescence was measured on-line in whole cells without addition of an aldehyde substrate. The resultant strain was also evaluated for its stability and fitness compared to those of the wild-type strain, F1.

Organisms and culture conditions. The strains used in these experiments are shown in Table 1. All cultures were grown at 28°C with appropriate antibiotic selection, except for *Escherichia coli* strains, which were grown at 37°C.

DNA isolation and manipulation. Large-scale plasmid DNA isolation was done by a modified alkaline lysis protocol (16). Chromosomal DNA was prepared by the protocol outlined by Ausubel et al. (2). All DNA preparations were further purified by CsCl-ethidium bromide ultracentrifugation (19). DNA modifications and restriction endonuclease digestions were performed as outlined by Sambrook et al. (19).

Transposon and plasmid construction. The transposon mini-Tn*5*Km*NX* was constructed with two 58-base oligonucleotides 5′ and 3′ with respect to the kanamycin resistance gene (Km^r) in pCR II (Invitrogen, San Diego, Calif.) (I end, 5′GGGCGCTAGCGAAATGTTGACTGTCTCTTGATCAGATCTTTCAATTCAGAAGAACTCG3′; O end, 5′CGAATTCTGACTCTTATACACAAGTTCTAGATTGCGGCCGCTTGGTTAAAAAATGAGC3′). Oligonucleotides were synthesized with a Beckman Oligo 1000 DNA synthesizer (Palo Alto, Calif.). Base substitutions were made to generate both I and O insertion sequences as well as unique *Not*I and *Xba*I sites inside the transposon for cloning. An extra adenine was mistakenly added between the *Not*I and *Xba*I sites in the O primer, but it did not affect the construction. Primers were used to amplify the kanamycin resistance gene from pCR II by using touchdown PCR (7). The manufacturer's protocol was used with the following thermocycler conditions. Initial denaturation at 94°C for 5 min, followed by five cycles of denatur-

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TABLE 1. Strains and plasmids used in this study

Plasmid or strain	Relevant genotype or characteristics	Source or reference
Plasmids		
pDTG514	pGem3Z with a 2.75-kb *Eco*RI-*Sma*I fragment from pDTG350 containing the *tod* promoter, P_{tod}; Ap^r	14
pUCD615	Promoterless *luxCDABE* cassette containing *ori* pSa and *ori* pBR322; Ap^r Km^r	18
pKK223-3	Expression vector containing the 5S ribosomal terminator *rrnB* T_1T_2	Pharmacia
pBSKS	pBluescript II KS^+ with MCS *Kpn*I-*Sac*I; Ap^r	Stratagene
pBSMCS(−)	pBluescript without MCS (*Bss*HII-*Bss*HII fragment removed); Ap^r	This study
pLJS	pBSMCS(−) with added *Xba*I, *Nhe*I, *Avr*II, and *Spe*I sites; Ap^r	This study
pLJS-*tod*	pLJS containing the 1.8-kb *Sma*I-*Xho*I *tod* promoter fragment from pDTG514; Ap^r	This study
pLJS-*lux*	pLJS containing the 8.35-kb *Kpn*I-*Pst*I *luxCDABE* cassette from pUCD615; Ap^r	This study
pLJST2	pLJS containing the 0.77-kb *Hin*dIII-*Hin*cII fragment from pKK223-3 cloned into *Hin*dIII-*Sma*I site; Ap^r	This study
pUC18Not	Cloning vector containing MCS flanked by *Not*I sites; Ap^r	11
pUC18Not-*lux*	Contains the 8.35-kb *Xba*I-*Pst*I fragment from pLJS-*lux*; Ap^r	This study
pUC18Not-*todlux*	Contains the 1.8-kb *Spe*I-*Xho*I fragment from pLJS-*tod*; Ap^r	This study
pUT	5.2-kb cloning vector containing *mob* RP4, *ori* R6K and Tn*5* *tnp* lacking *Not*I sites; Ap^r	11
pCR II	3.9-kb cloning vector for PCR products with 3′ A overhangs; Ap^r Km^r	Invitrogen
pUTK209	pCR II containing mini-Tn*5*Km*NX* with unique *Not*I and *Xba*I sites; Ap^r Km^r	This study
pUTK210	pUT containing mini-Tn*5*Km*NX*; Ap^r Km^r	This study
pUTK211	pUT/mini-Tn*5*KmT2 containing the 0.8-kb *Not*I-*Avr*II *rrnB* T_1T_2 fragment; Ap^r Km^r	This study
pUTK214	pUT/mini-Tn*5*Km*tod-lux* containing the 10.2-kb *Not*I-*Xba*I fragment from pUC18Not-*todlux*; Ap^r Km^r	This study
Strains		
E. coli		
DH5α	F^- φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Gibco BRL
S17-1(λ*pir*)	λ*pir recA thi pro hsdR* M^+ RP4:2-Tc:Mu:Km Tn*7*Tp^rSm^r; mobilizing strain for pUT/mini-Tn*5* derivatives	6
INVαF′	Strain used with TA cloning vector, pCR II; F′ φ80*lacZ*αΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Invitrogen
P. putida		
F1	Contains a chromosomally encoded *tod* operon for toluene degradation	28
TVA8	F1 containing a mini-Tn*5*Km*tod-lux* insertion in the chromosome; Km^r	This study

ation at 94°C for 1 min, 72°C annealing for 1 min, and 72°C extension for 2 min. The annealing temperature was then lowered 5°C every five cycles until 42°C, at which point, eight cycles were run, followed by a final extension of 15 min at 72°C. The resultant PCR fragment was cloned into the transposon delivery vector pUT, generating pUTK210. The cloning vector pLJS was constructed from pBluescript II (KS) (Stratagene, LaJolla, Calif.) by cleavage with *Bss*HII removing the multicloning site (MCS). The resultant plasmid was named "pBSMCS(−)." Two oligonucleotides (a 47-mer and a 44-mer) (*Kpn*I end, 5′CCAAGCGCGCAACTAGTCTAGACTAAAG CTAGCCTAGGCTGGGATCC3′; *Sac*I end, 5′GTGAGCGC GCGTAATACGAGCTAGCCTAGGGCGAATTGGAGCA C3′) were synthesized to regenerate the MCS and add the restriction sites *Xba*I, *Nhe*I, *Spe*I, and *Avr*II. The orientation of the added sites can be seen in Fig. 1. The new MCS was amplified from pBluescript II (KS) by using the manufacturer's protocol with the following thermocycler conditions. Initial denaturation was at 94°C for 5 min, followed by 38 cycles of denaturation at 94°C for 30 s, annealing at 42°C for 1 min, extension at 72°C for 30 s, and final extension at 72°C for 15 min. The amplified fragment was cleaved with *Bss*HII, ligated into pBSMCS(−), and transformed into DH5α. A portion of pLJS was sequenced, confirming the base substitutions and integrity of the MCS, with an Applied Biosystems model 373A sequencer (Foster City, Calif.). Plasmid pLJST2 was generated by directional cloning of the 0.8-kb *Hin*dIII-*Hin*cII fragment containing the 5S ribosomal *rrnB* T_1T_2 transcription terminator from pKK223-3 (Pharmacia, Piscataway, N.J.) into pLJS cleaved with *Hin*dIII and *Sma*I. The *Not*I-*Avr*II terminator fragment from pLJST2 was subsequently cloned into the *Not*I-*Xba*I site of pUTK210, yielding pUTK211 containing mini-Tn*5*KmT2. This allowed for the subsequent destruction of the *Xba*I site by heterologous ligation and the regeneration of the *Not*I and *Xba*I unique sites downstream of the terminator. Mini-Tn*5*Km*tod-lux* (pUTK214) was generated by directional cloning of the 10.2-kb *Not*I-*Xba*I *tod-lux* fragment from pUC18Not-*todlux* (Table 1) into the *Not*I-*Xba*I site of pUTK211. Both the insert and vector DNA were purified by agarose gel electrophoresis and electroeluted prior to cloning. Electrocompetent *E. coli* S17-1(λ*pir*) cells were prepared and ligations were electroporated as outlined by the manufacturer (BTX, San Diego, Calif.). All other plasmids and relevant constructs are described in Table 1.

Strain construction. Plasmid pUTK214 was conjugated into *P. putida* F1 from *E. coli* S17-1(λ*pir*) as previously described (6). Strains carrying transposon insertions were selected on *Pseudomonas* isolation agar (Difco, Detroit, Mich.) supplemented with 50 μg of kanamycin/ml. Colonies which produced light upon exposure to toluene were grown in mineral salts media (MSM) (23) with toluene vapor to ascertain that the transposon had not inserted into a gene required for cell

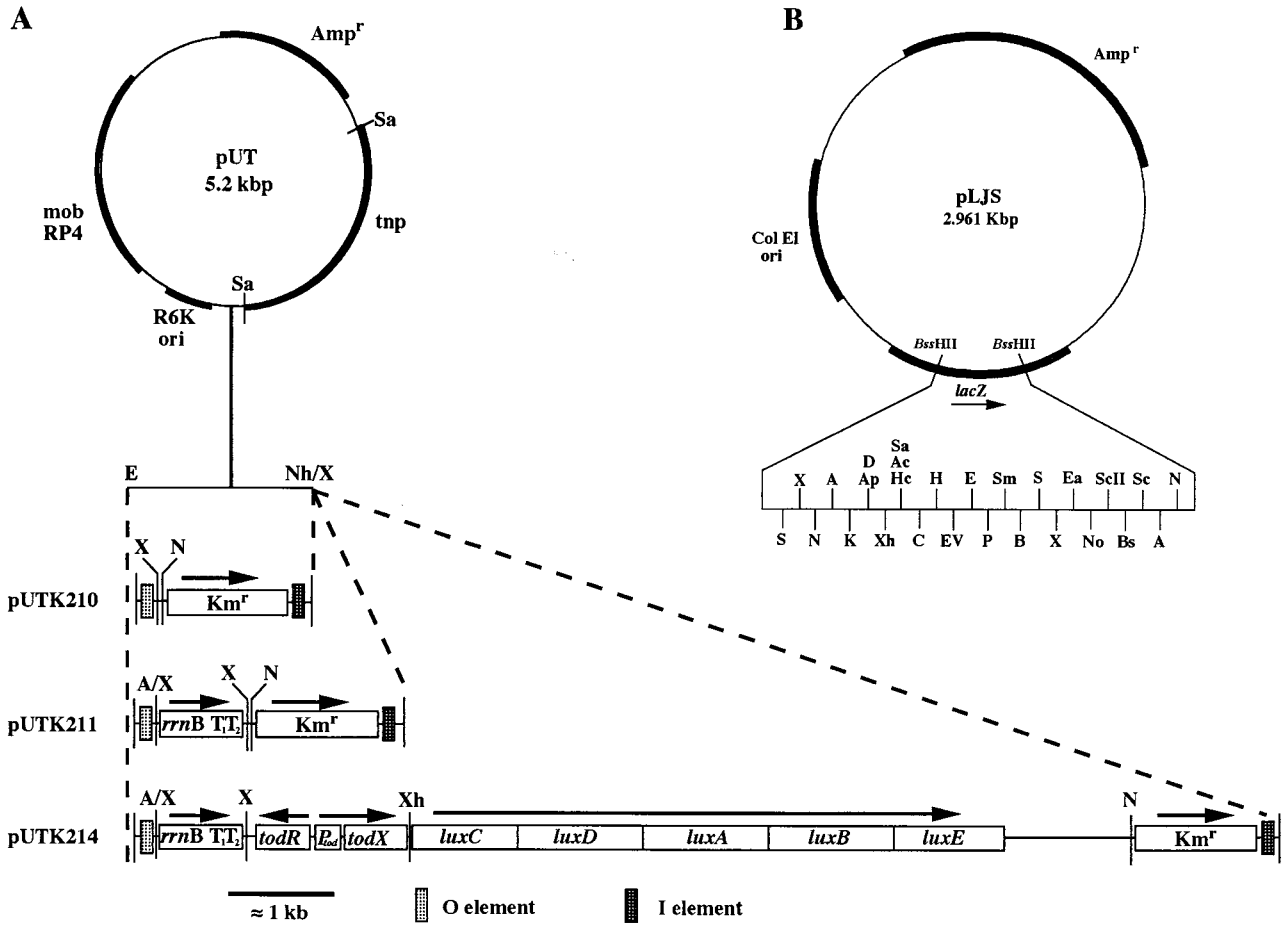

FIG. 1. (A) Construction of mini-Tn*5*Km*tod-lux*. A/X and Nh/X represent the *Avr*II-*Xba*I and *Nhe*I-*Xba*I heterologous cloning sites, respectively. N, *Not*I; Sa, *Sal*I; X, *Xba*I. (B) Cloning plasmid pLJS with unique restriction sites. A, *Avr*II; Ac, *Acc*I; Ap, *Apa*I; B, *Bam*HI; Bs, *Bst*XI; C, *Cla*I; D, *Dra*II; E, *Eco*RI; Ea, *Eag*I; EV, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; K, *Kpn*I; Nh, *Nhe*I; P, *Pst*I; S, *Spe*I; Sc, *Sac*I; ScII, *Sac*II; Sm, *Sma*I; Xh, *Xho*I.

growth and also to evaluate their performance as bioreporters in liquid, growing-cell assays (8). A strain designated TVA8 was selected for further study and subjected to DNA-DNA hybridization to verify transposition, as opposed to recombination, by using a ^{32}P-labeled probe specific for the Tn*5* transposase (*tnp*) contained on pUT. Equal target amounts of *luxA*, *todC*, and *tnp* DNA were loaded onto a Biotrans nylon membrane (ICN, Irvine, Calif.) by using a Bioslot blot apparatus (Bio-Rad, Hercules, Calif.) according to the manufacturer's protocol. The blot consisted of chromosomal DNA from F1, TVA8, and the aforementioned controls. The DNA was loaded in triplicate, the blot was subdivided, and each separate blot was hybridized with either *luxA*, *todC*, or *tnp* PCR-generated ^{32}P-labeled DNA probes. Blots, hybridized and washed as previously described (1), verified that TVA8 contained *luxA* and *todC* but not *tnp* (data not shown). The negative transposase result confirmed that transposition had occurred.

Stability assays. Batch stability assays were performed by transfer of 1 ml of a 100-ml overnight culture grown in Luria-Bertani (LB) broth with 50 μg of kanamycin/ml ($LBKm_{50}$) to a 250-ml Erlenmeyer flask with toluene used as the sole carbon source (supplied as vapor). One milliliter of culture was transferred every day for 5 days to flasks containing 100 ml of MSM supplied with toluene vapor (without Km_{50}). Assays were performed in triplicate. Before each transfer, cells were plated on selective ($LBKm_{50}$) and nonselective (LB) media to ascertain loss of kanamycin resistance resulting from deletion or excision of the transposon. Colonies were subjected to colony hybridization with a 295-bp *luxA* DNA probe (12). Stability was also assayed in continuous culture with a New Brunswick Bio Flow fermentor (Edison, N.J.) with a 370-ml vessel operated at 28°C at 180 rpm. The feed consisted of MSM supplemented with toluene at approximately 100 mg/liter at a flow rate of 1.0 ml/min. Toluene was fed by simultaneously adding toluene-saturated MSM at a flow rate of 0.2 ml/min and MSM at a flow rate of 0.8 ml/min by using FMI metering pumps (Oyster Bay, N.Y.). The fermentor was operated for 14 days (100 generations) with daily bioluminescence and optical density (OD) measurements. Plate counts (at 7 and 14 days) from selective ($LBKm_{50}$) and nonselective (LB) media were compared to determine if the kanamycin marker was being lost, and *luxA* colony blot hybridization was performed to confirm that all colonies contained the *lux* transposon insert. In batch and chemostat stability studies, TVA8 did not demonstrate instability when subjected to the same evaluation. From batch assays, the selective/nonselective plate count ratio was 1.12 ± 0.13 after five daily transfers, and all colonies hybridized with the *luxA* probe. After a 14-day continuous cultivation, the selective/nonselective plate count ratio was 1.05 ± 0.13, and all colonies from selective and nonselective plates were *lux* positive.

Comparison of growth of TVA8 and F1 on toluene. To examine the effect of bioluminescence on the fitness of TVA8, growth curves of TVA8 and F1 were obtained by growing cells

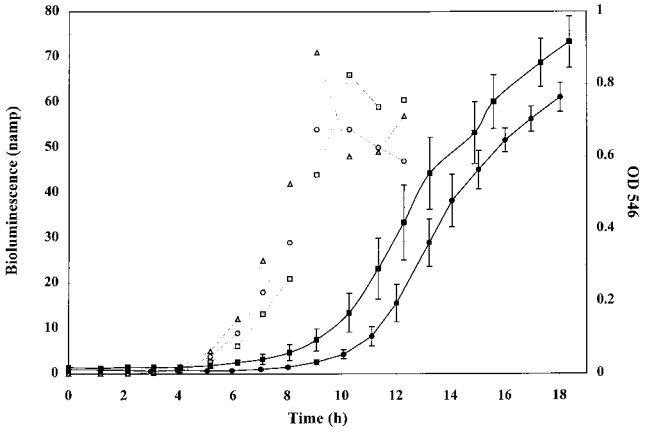

FIG. 2. Bioluminescence and growth of TVA8 and growth of F1 on toluene vapor under batch conditions. ○, □, and △ represent individual replicates of bioluminescence readings over time. The solid squares (TVA8) and circles (F1) represent the average OD_{546} of three replicates.

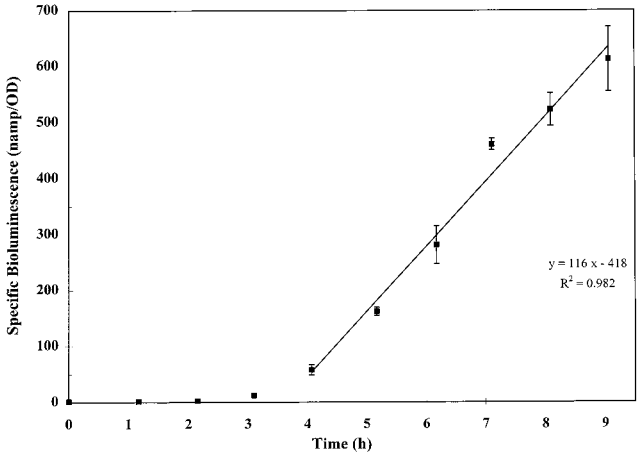

FIG. 3. Specific bioluminescence of TVA8 grown on toluene vapor. The regression line equation is $y = 116x - 418$ ($r^2 = 0.982$). (The first four time points were not included in the linear regression because the organisms were in lag phase.)

in 100 ml of MSM in 250-ml Erlenmeyer flasks with toluene vapor supplied as the sole carbon source. Flasks were inoculated from a fresh overnight culture, grown to an OD at 546 nm (OD_{546}) of 1.0 in 100 ml of LB, washed twice in 100 ml of MSM, and resuspended in 100 ml of MSM. A 1-ml aliquot of this suspension was added to the toluene flasks. The cultures were shaken at 200 rpm at 28°C and sampled approximately every hour. The OD_{546} was measured for each culture, and rates of increase in OD were determined from the linear portion of the curves. Growth curves for TVA8 and the parent strain F1 on toluene vapor are shown in Fig. 2. The curves show similar shapes with different lag times for TVA8 and F1 that can be attributed to slightly different inoculum concentrations. Rates were computed from the slopes of the linear portion of the growth curve for both strains. The average rates of increase in OD for F1 and TVA8 were (2.1 ± 0.3) and (2.2 ± 0.3) $min^{-1} \times 10^{-3}$, respectively and were not statistically different ($\alpha = 0.05$). These results demonstrate that the bioluminescence reactions do not appear to affect cell growth.

Bioluminescence of TVA8 was measured during growth on toluene and is shown along with the cell density data in Fig. 2. The graph shows that there is a relationship between an increase in biomass and an increase in light production. At higher cell densities, cells likely became limited for oxygen, resulting in decreased bioluminescence. Specific bioluminescence (nanoamperes/OD_{546}) of TVA8 versus time shows an increase in specific bioluminescence (Fig. 3). This suggests that a steady state of luciferase in the cell is not obtained in this time frame and that luciferase is accumulating (Fig. 3).

Bioluminescence response of TVA8 to toluene, BTEX compounds, and JP-4 jet fuel. Bioluminescence assays were conducted as described by Heitzer et al. (8). Aqueous solutions of toluene, benzene, ethylbenzene, phenol, isomers of xylene, and JP-4 jet fuel constituents were prepared by adding pure hydrocarbon or JP-4 to MSM in a 1:10 (vol/vol) ratio. The solutions were placed on a rotary shaker for 24 h. After phase separation, aqueous-phase aliquots were added to test vials. Final concentrations of dissolved toluene in growing-cell assays ranged from 0 to 50 mg/liter (based on water solubility). The final concentration of the other hydrocarbons was 50 mg/liter (based on their water solubility), and the percentage of JP-4-saturated MSM in the test samples was 2%. Vials containing test solutions and cells were shaken at 150 rpm on a rotary shaker, and bioluminescence was measured every 30 min. Sample vials were placed in a light-tight box, and light output was measured with a liquid light pipe and an Oriel photomultiplier and digital display (models 77340 and 7070; Stratford, Conn.) (8). The light detection methods for continuous culture and growth curves were similar, except that the light-tight box was modified to hold a cuvette, allowing for light measurements and OD readings to be taken consecutively.

In preliminary experiments, an incubation time of 2 h was shown to provide a consistent light response and signal intensity. After 2 h, the final OD_{546} was measured, and values were expressed as specific bioluminescence (nanoamperes/OD_{546}). An increase in bioluminescence was observed to correlate with increasing toluene concentrations (Fig. 4). The bioluminescence response to toluene concentrations over the range of 5 to 20 mg/liter was linear, with specific bioluminescence values of 133 to 228 nA/OD_{546}. The fold increase in light response for concentrations above 20 mg/liter was less, showing a specific bioluminescence value of 290 nA/OD_{546} at 50 mg of toluene per liter. The overall bioluminescence response curve exhib-

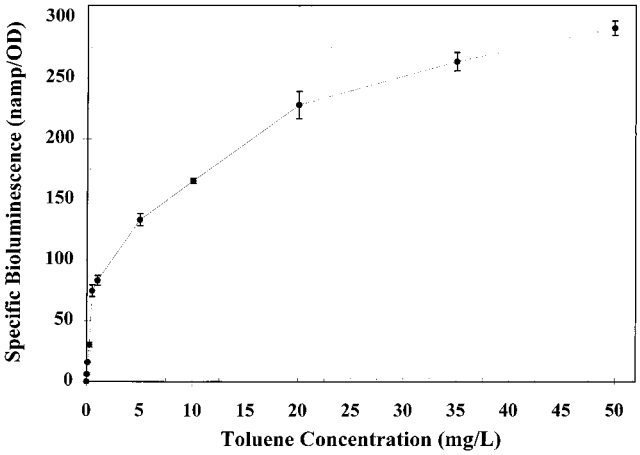

FIG. 4. Bioluminescence response of TVA8 to increasing concentrations of toluene after a 2-h exposure. Values are averages of three replicates and have been normalized to the cell density (OD_{546}).

TABLE 2. Effect of BTEX, phenol, and JP-4 constituents on the bioluminescence response of TVA8

Treatment[a]	Exposure time (h)	Specific bioluminescence (nA/OD)[b]
Buffer (control)	2	0.2 ± 0.1
Toluene	2	234 ± 7
Benzene	2	242 ± 9
Ethylbenzene	2	1.0 ± 0.2
	4	47 ± 6[c]
o-Xylene	2	0.5 ± 0.1
m-Xylene	2	38 ± 3
p-Xylene	2	24 ± 2
Phenol	2	70 ± 2
JP-4	2	93 ± 4

[a] Final concentration for BTEX and phenol treatments was 50 mg/liter (based on water solubilities), and they were added as a hydrocarbon-saturated MSM solution. The final percentage of water-soluble JP-4 constituents was approximately 2%.

[b] Values are averages ± standard deviations of three replicate samples. Values were normalized to the final cell density (OD_{546}).

[c] The value for the 4-h reading was measured from a similar but separate experiment.

ited Michaelis-Menten kinetics, showing saturation at higher toluene concentrations. The toluene detection limit was determined to be 30 μg/liter (threefold increase over background bioluminescence). There was a significant light response to benzene, *m*- and *p*-xylenes, phenol, and JP-4 (Table 2). The same concentrations of toluene and benzene (50 mg/liter) resulted in a similar light response. There was no increase in bioluminescence upon exposure to *o*-xylene. The light response due to JP-4 was significantly greater than the additive responses for JP-4 components (i.e., BTEX compounds) present at their estimated concentrations in water saturated with JP-4 (22). The increased response may be the result of induction due to other components of JP-4 which were not tested. A significant light response was observed for ethylbenzene after 4 h. After a 2-h incubation, the cell densities for the ethylbenzene treatments were significantly less than those for the other samples, indicating that there may have been a toxicity effect. Further experiments showed that 50 mg of ethylbenzene per liter would induce the bioluminescence response without a lag period when cells were previously grown on ethylbenzene and then subjected to growing-cell assays.

Conclusions. The majority of bioluminescent reporter systems currently being used are the result of cloning of a promoter in front of either a promoterless *luxAB* or *luxCDABE* gene cassette and transfer of the plasmid construct into the strain that contained the particular promoter. Plasmid-based systems have obvious drawbacks, such as the need for constant selective pressure to ensure plasmid maintenance (17). Another important consideration is plasmid copy number. In a positively regulated system, copy number can negatively effect gene expression. Multiple copies of the promoter binding region for the regulatory protein on the plasmid compete with the binding site on the chromosome, causing less expression of the operon being studied (27). This negative effect is important when *lux* fusions are used for on-line monitoring of bacterial processes.

TVA8 was capable of growth on MSM with toluene as a sole carbon source, demonstrating that the transposon insertion did not disrupt a gene necessary for growth. Furthermore, TVA8 did not show loss of the transposon insertion or loss of bioluminescence after 100 generations in continuous culture or five successive transfers in batch culture without antibiotic selection. These results indicate that selective pressure is not necessary for strain integrity. This stability is important, since it eliminates the need for antibiotic selection, which, if required, would exclude the use of this bioreporter in situ. TVA8 was also compared to the wild-type strain, F1, to ascertain whether or not the bioluminescent reporter incurred a significant metabolic demand on the cell, as well as whether the site of transposition was a hindrance to the cell. No difference in growth between the two strains was seen, suggesting that neither the insertion site nor the *lux* fusion was a significant handicap to the cell.

The *tod-lux* reporter was highly sensitive, detecting 30 μg of toluene/liter. This bioreporter also showed a very low background level of bioluminescence (less than 1 nA/OD_{546}), demonstrating its usefulness for detecting toluene present at low concentrations in aqueous solutions. Significant light levels were observed for very low ODs (Fig. 2).

TVA8 can be described as a generalized BTEX bioreporter rather than simply a toluene bioreporter, since it was responsive to benzene, ethylbenzene, and *m*- and *p*-xylene and can therefore be used as a bioreporter for hydrocarbon contamination for fuels containing BTEX compounds. TVA8 can also be used for on-line monitoring of trichloroethylene cometabolism, since the *lux* and *tod* operons are under the same regulation (toluene dioxygenase catabolizes trichloroethylene).

Bioluminescent reporters may have great potential for field use applications, since they can provide on-line and nondestructive analyses of gene expression as well as detection of chemical contaminants. The development of stable transposon insertions of *lux* reporter gene fusions into environmental isolates expands the utility of bioreporter strains for in situ sensing of gene expression.

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