



Pseudomonas putida B2: a *tod-lux* bioluminescent reporter for toluene and trichloroethylene co-metabolism

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A *tod-lux* transcriptional fusion bioluminescent reporter strain, *Pseudomonas putida* B2, was developed to permit on-line analysis of trichloroethylene (TCE) transformation by toluene dioxygenase (*todC1C2BA*) in *Pseudomonas putida* F1. Strain B2 was exposed to toluene in growing and resting cell bioluminescence assays. The growing cells showed a direct correlation between bioluminescence and toluene concentration, while resting cells showed reproducible bioluminescence with repeated toluene exposures. In addition, *P. putida* B2 was encapsulated in alginate beads and used in a packed bed flow-through differential volume reactor. The TCE feed into the differential volume reactor was constant at 20 mg L⁻¹ and toluene was pulsed in square-wave perturbations at 10 mg L⁻¹. The system showed a direct correlation between the expression of the *tod* operon (as monitored by light output) and the co-metabolism of TCE. Approximately 20% of the TCE and 50% of the toluene was removed at a flow rate of 0.4 ml min⁻¹. This approach allowed the on-line monitoring of *tod* gene expression and its relation to TCE biotransformation.

Keywords: TCE; toluene; jet fuel; bioluminescence; *lux*; co-metabolism; *Pseudomonas putida*

Introduction

The environmental fate and bioremediation potential of trichloroethylene (TCE) have received considerable attention due to its extensive production, use [25] and occurrence as a groundwater priority pollutant of toxic and carcinogenic concern [4,18,22,26,32]. Bacterial metabolism of TCE has been extensively reviewed [2]. TCE degradation is co-metabolic in that TCE is not used as a carbon source but is fortuitously degraded. Due to the potential production of carcinogenic vinyl chloride during anaerobic degradation [13], much of the recent focus on TCE biodegradation has been on aerobic, oxygenase-mediated TCE co-metabolism [16,17]. Substantial information has been developed on monooxygenase-mediated co-metabolism of TCE [17,28] with particular emphasis on the methane monooxygenases and a variety of toluene monooxygenases.

Toluene degradation occurs via catabolic pathways containing both monooxygenases and dioxygenases which have the ability to oxidize TCE [2,23,27]. The toluene dioxygenase (*todC1C2BA*) contained in *Pseudomonas putida* F1 is also capable of transforming TCE [33].

Central to the use and further development of aerobic co-metabolic TCE bioremediation is the ability to monitor, control and optimize such biodegradative processes. One such strategy has been the development of bioluminescent *lux* gene fusions for use in on-line reporter technology [1,10]. The use of *lux*-reporter systems in the study of the

on-line monitoring of naphthalene degradation has been well documented [6,10]. These reporter systems have also been used to assess the bioavailability of pollutants to catabolic organisms [7,10]. However, little work has been done using reporter strains in the co-metabolism of pollutants.

The objective of this work is to extend the application of *lux* reporter technology to monitoring and optimizing the co-metabolic oxidation of pollutants such as TCE. For this purpose the *tod* system contained in *P. putida* F1 was chosen to develop a *tod-lux* gene fusion to monitor the expression of toluene dioxygenase.

Materials and methods

Strain construction

The strains and plasmids used in this study are shown in Table 1. *Escherichia coli* was grown in Luria-Bertani (LB) broth and on LB agar plates at 37°C. *Pseudomonas putida* F1 was grown on yeast extract-peptone-glucose (YEPG) medium consisting of 0.2 g yeast extract, 2.0 g polypeptone, 1.0 g D-glucose and 0.2 g ammonium nitrate (pH 7.0) in 1 L of distilled water at 28°C.

One-liter cultures of *E. coli* JM109 and HB101 harboring the appropriate plasmids were harvested and plasmid DNA was isolated using a modified alkaline lysis procedure [19]. The plasmid DNA was subjected to CsCl density gradient purification, followed by butanol extraction and ethanol precipitation [21]. Plasmid DNA was resuspended in TE buffer (10 mM Tris-base, 1 mM EDTA, pH 8.0) and stored at 4°C until used. Restriction endonucleases and T4 DNA ligase were obtained from Gibco BRL (Gaithersburg, MD, USA) and used according to manufacturers' protocols. Cloning techniques were performed as outlined in Sambrook *et al* [21]. The reporter plasmid pUTK30 was gener-

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Table 1 Strains and plasmids used in this study

Strain	Plasmid	Relevant characteristic(s)	Reference
<i>E. coli</i> JM109	pDTG514	pGem3Z with a 2.75-kb <i>EcoRI-SmaI</i> fragment from pDTG350 containing the <i>tod</i> promoter, Amp ^R	[14,15]
<i>E. coli</i> HB101	pUCD615	Promoterless <i>luxCDABE</i> cassette, mob ⁺ Amp ^R , Km ^R	[20]
<i>P. putida</i> F1	none	Contains chromosomally-encoded <i>tod</i> operon for toluene degradation	[27]
<i>P. putida</i> B2	pUTK30	<i>Tod-lux</i> reporter containing the <i>tod</i> promoter fragment inserted upstream of the promoterless <i>luxCDABE</i> cassette, Amp ^R , Km ^R	this study
<i>E. coli</i> DF1020	pRK2013	Helper plasmid, Amp ^R , Km ^R , Tra ⁺	[3]

ated by cloning the *tod* promoter [12,29] from pDTG514 [14,15] in front of the *lux* gene cassette of pUCD615 [20]. This was accomplished by directionally cloning a 2.75-kb *EcoRI-XbaI* fragment from pDTG514 into an *EcoRI-XbaI* digest of pUCD615 (Figure 1). Transformations were performed using subcloning efficiency competent cells (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer's protocol. Transformants were selected on LB plates containing 50 $\mu\text{g ml}^{-1}$ kanamycin. Plasmid minipreps of transformants were performed as described by Holmes and Quigley [9] and cleaved with *Bam*HI to confirm insertion of the *tod* fragment. The resultant *E. coli* strain, JBF-7 harbored the reporter plasmid pUTK30.

Triparental matings were carried out using a modified version of the filter technique. Pure cultures of donor (JBF-7, pUTK30), helper (DF1020, pRK2013 [3]), and recipient (F1) were grown to an optical density at 546 nm (OD_{546}) of approximately 1.0 in LB broth with appropriate antibiotics. Cells were harvested by centrifugation at $9800 \times g$ for 10 min. The pellets were suspended and washed three times in 100 ml 50 mM KH_2PO_4 (pH 7.0), and suspended in 50 ml 50 mM KH_2PO_4 .

The three strains were mixed using a ratio of 2 : 1 : 1 (donor/helper/recipient). The cell suspension was filtered through a Teflon membrane (47 mm, 0.22 μm pore size) and the filter was placed on a LB plate. After overnight incubation at 28°C, the filter was removed and washed in 1.5 ml 50 mM KH_2PO_4 . Serial dilutions were performed and dilutions were plated onto *Pseudomonas* Isolation Agar plates (Difco, Detroit, MI, USA). After 48 h incubation, toluene vapor was introduced and colonies which produced light were selected for further characterization. One of five kanamycin-resistant strains which emitted light in response to toluene vapor, *P. putida* B2, was chosen for use in the remaining experiments.

Bioluminescence analysis

In the batch and reactor studies, bioluminescence was measured using a photomultiplier, which converts the light to an electric current. The photomultiplier in the resting cell assays and the reactor system was connected to a computer and bioluminescence as namps current was recorded.

Batch experiments

Assays of growing cells were conducted as described by Heitzer *et al* [7]. An overnight culture from a frozen stock of *P. putida* B2 was prepared in a 250-ml Erlenmeyer flask containing 100 ml LB and 50 $\mu\text{g ml}^{-1}$ kanamycin. A sub-culture was prepared and cells were used in mid-log phase

(OD_{546} of 0.45–0.48). A 2.5-ml aliquot was added to 2.5 ml mineral salts medium (MSM) containing 0–50 mg L^{-1} toluene or 10–100 μl of JP4 jet fuel-saturated MSM. The concentration of toluene in water saturated with JP4 jet fuel is approximately 8 mg L^{-1} [24]. Bioluminescence was measured every 30 min [7].

Cells for resting cell assays were grown on MSM supplemented with 2.7 g L^{-1} succinate. A culture of *P. putida* B2 was harvested at an OD_{546} of approximately 0.8. The cells were centrifuged at $15000 \times g$ for 10 min, and resuspended in MSM to OD_{546} of 0.6. Four milliliters of culture were added to each of six 26-ml vials with Mininert valves (Dynatech, Chantilly, VA, USA) with stir bars. One vial was used for multiple toluene exposures, while the remainder were used for single exposures. The vials were magnetically stirred in a light-tight sampling cell. Toluene-saturated MSM and MSM alone were added to yield an OD_{546} of 0.47, and 10 mg L^{-1} toluene was injected six times over a 130-h period for the multiple-exposure vial. At the same time points, a single-exposure vial was injected with 10 mg L^{-1} toluene. The light response was recorded every 3 min with a photomultiplier connected to a data acquisition computer [10].

Immobilized cell reactor system

P. putida B2 was encapsulated in alginate beads for the immobilized cell reactor system. Cells were grown in 1 L LB to an OD_{546} of 1.2 and were centrifuged at $5500 \times g$ for 10 min, washed three times in 0.9% NaCl and suspended in 40 ml 0.9% NaCl. The cell suspension was added to 80 ml of an alginic acid solution (28 g L^{-1} low viscosity alginate, 0.9% NaCl) [31]. The cell-alginate suspension was placed in a 60-ml syringe, forced through a 25-gauge needle, and allowed to drop into a 0.5 M CaCl_2 solution. The alginate was cross-linked by the Ca^{2+} ions, thus encapsulating the cells. The cells were subsequently placed in a fresh solution of 0.1 M CaCl_2 and allowed to sit for 30 min prior to use.

A differential volume reactor (DVR) was used to simulate a section of an ideal plug flow reactor. Influent to the reactor was dispersed through a porous metal frit to provide a flat velocity profile to the bed. The reactor measured 5.0 cm i.d. \times 5.0 cm long. A complete description of this reactor can be found in Webb *et al* [30]. In this investigation, a system was designed incorporating the DVR as illustrated in Figure 2. The system was equipped with three Millipore (Bedford, MA, USA) stainless steel substrate containers rated to 690 kPa. The feed from the substrate vessels to the reactor inlet was controlled by two Gilson (Middleton, WI, USA) 301 HPLC pumps. A flow rate of

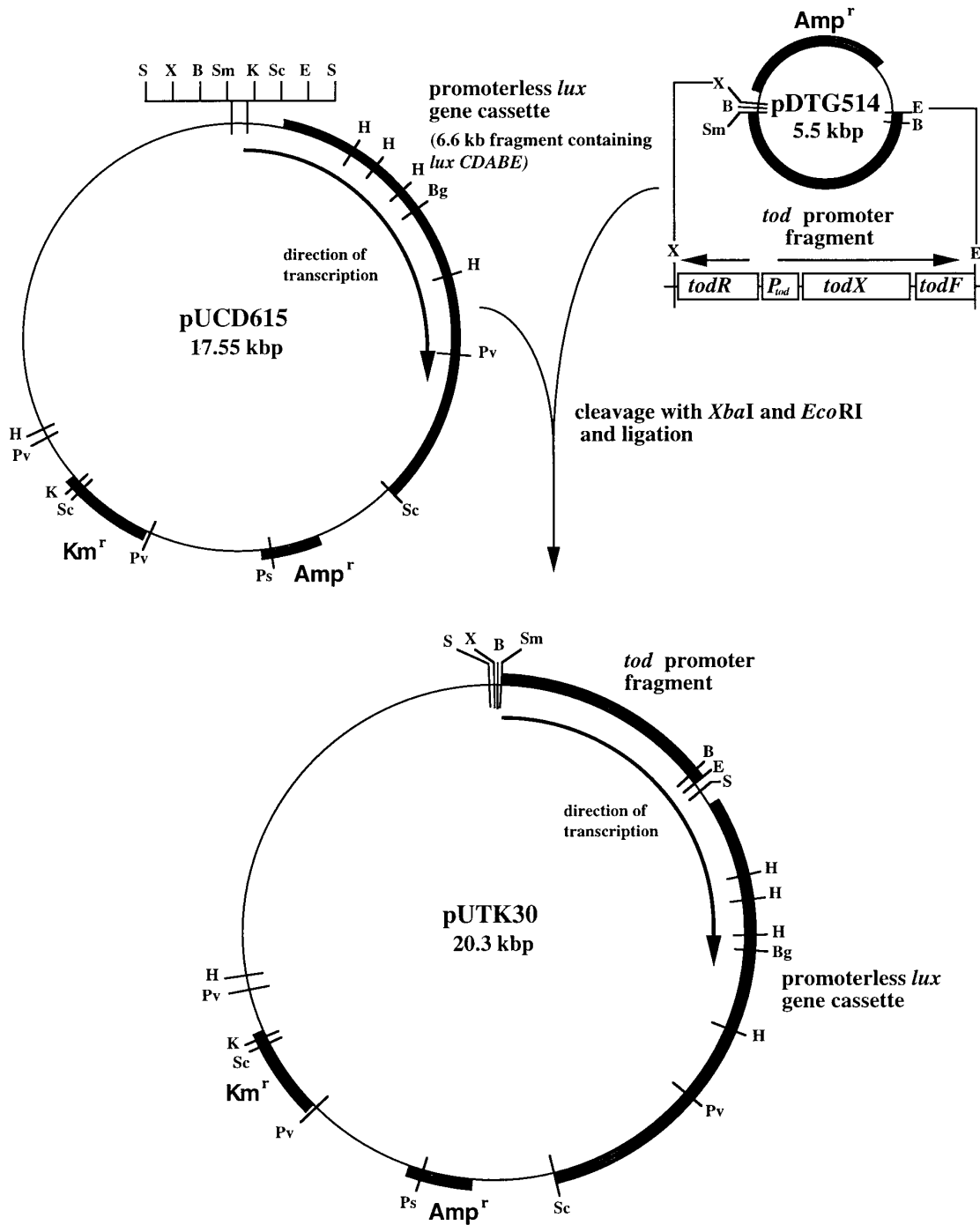


Figure 1 Construction of the *tod-lux* reporter plasmid pUTK30. The 2.75-kb *EcoRI-XbaI* fragment from pDTG514 [14,15] was cloned in front of the promoterless *lux* gene cassette in pUCD615 [20]. Abbreviations: B, *Bam*HI; Bg, *Bgl*III; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*II; Sc, *Sac*I; S, *Sal*I; Sm, *Sma*I; X, *Xba*I.

0.4 ml min⁻¹ was maintained. The substrate vessels were pressurized with oxygen to provide the system with an electron acceptor. All medium vessels contained trace mineral medium [11] with the addition of 3 mg L⁻¹ pyruvic acid and a 0.1 M solution of Tris-base (pH 7.0). Phosphate buffers were not used since phosphate ions complex with Ca²⁺ ions and disrupt the alginate crosslinking. In addition to this medium, two of the vessels contained either TCE or

toluene. The inlet concentration of toluene was altered by using square-wave perturbations with 20-h cycles (10 h with toluene, 10 h without toluene) using an HPLC pump controlled by a timer. During feed portions of the cycle, 10 mg L⁻¹ toluene was introduced into the inlet of the reactor. The inlet TCE concentration was constant at 20 mg L⁻¹.

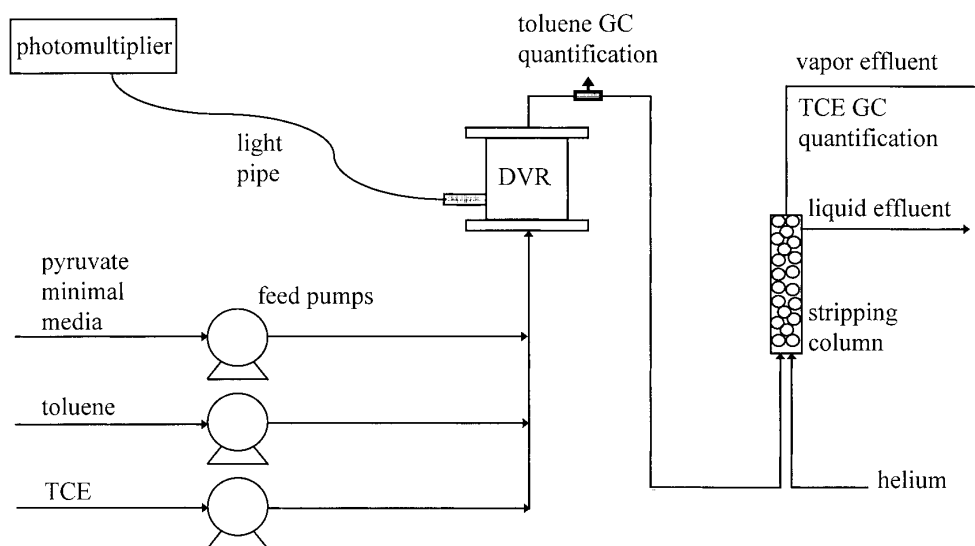


Figure 2 Diagram of the on-line DVR system used to monitor the co-metabolism of TCE.

TCE and toluene analysis

Analysis of TCE in the reactor effluent was performed on-line using a stripping column (12.5 cm length and 0.4 cm inner diameter) packed with 3-mm glass beads to provide adequate surface area for TCE separation. TCE was stripped with helium, the GC carrier gas. The stripping column outlet was attached to a gas chromatograph (GC, Hewlett Packard (Wilmington, DE, USA) (HP) 5890 Series II) with an electron capture detector by a heated sample line maintained at 75°C. Automatic injections (25 μ l) were made by a computerized control process (HP Chem Station software). The GC was equipped with a cross-linked methyl silicone capillary column (length 30 m, i.d. 0.2 mm, 0.33- μ m film thickness) while the oven was operated isothermally at 60°C. Other operating parameters included an injection temperature of 150°C, detector temperature of 200°C and a split ratio of 10 : 1. This system was equipped with a bypass line around the reactor in order to calibrate the stripping column.

Toluene samples were removed at 0.5-ml aliquots from the effluent sampling port (Figure 2) and injected into 1.5 ml sample vials. Headspace analysis was performed using a Shimadzu (Columbia, MD, USA) GC-9A gas chromatograph equipped with a 2.44-m, 3.2-mm diameter Poropak N packed column and a flame ionization detector. The isothermal temperature of the oven was 210°C, and both the detector and injector temperatures were 220°C.

Results

Batch experiments

Assays of growing cells showed an increasing bioluminescent response with increasing concentrations of toluene, up to 10 mg L⁻¹ toluene, after 90 min exposure (Figure 3). The relationship was linear over this range. The bioluminescent response varied from 2.4 namp at 0.1 mg L⁻¹ toluene to approximately 90 namp for 10, 20 and 50 mg L⁻¹ toluene. There was not a significant bioluminescent response for 0 and 0.01 mg L⁻¹ toluene (data not shown). Similarly, the light response increased with increasing concentration of

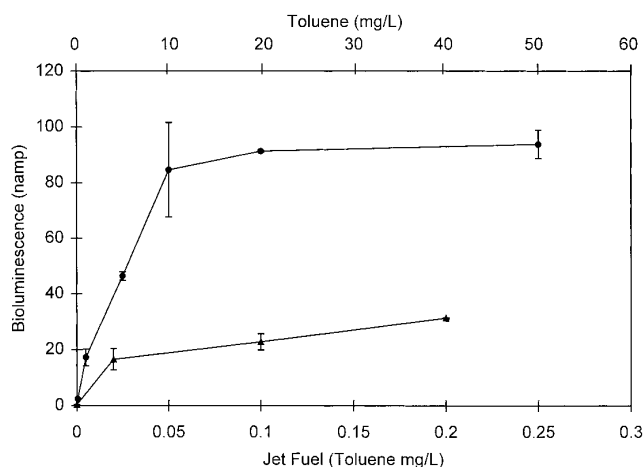


Figure 3 Bioluminescent response to varying concentrations of toluene (●) and JP4 jet fuel, expressed as mg L⁻¹ toluene (▲) in growing cell assays after a 1.5-h exposure.

water-soluble jet fuel components (Figure 3). At 10 μ l jet fuel-saturated MSM added (approximately 0.02 mg L⁻¹ toluene), the light response was 16 namp, while at 100 μ l added (approximately 0.2 mg L⁻¹ toluene), the response increased to 31 namp. The bioluminescent response for the 0.1 mg L⁻¹ toluene equivalent of jet fuel was about 10 times that for 0.1 mg L⁻¹ toluene, so other components beside toluene appear to have affected bioluminescence.

In resting cell assays, the bioluminescent response to single exposures of toluene was rapid and reproducible (Figure 4). The initial injection to the multiple exposure vial showed the same characteristic light response as each single exposure vial. However, there was a slower response (the rate of increase in bioluminescence, h⁻¹) upon initial exposure to toluene compared with the response of cells previously exposed to toluene. In addition, the response rate increased with each exposure to toluene (Table 2). However, the maximum bioluminescent response for both the single and multiple exposures was the same at 573 \pm 127 namp.

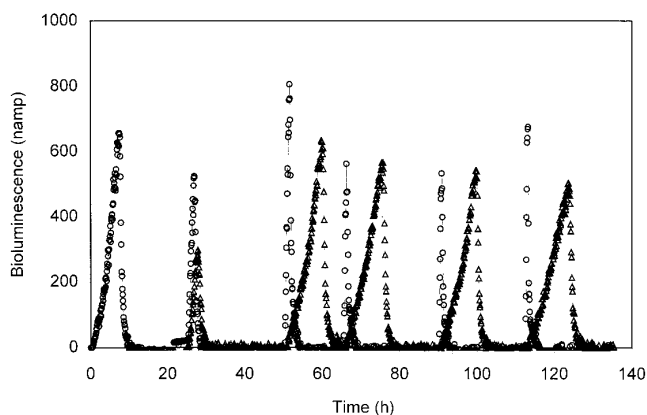


Figure 4 Bioluminescent response to multiple and single exposures of 10 mg L^{-1} toluene by resting cells of *P. putida* B2 in batch studies. Symbols: \circ multiple exposure, Δ single exposure.

Table 2 Bioluminescent response rate (namp h^{-1}) for multiple and single exposures of 10 mg L^{-1} toluene^a

Time point	Multiple exposure vial ^b	Single exposure vials ^c
1	95	ND
2	321	137
3	642	67
4	768	60
5	737	60
6	973	49

^aResponse rate is defined as rate of bioluminescence increase with time.

^bA single vial, with multiple additions of toluene.

^cA new vial, previously unexposed to toluene, injected with toluene at each time point.

ND, not done.

Immobilized cell reactor system

The DVR system loaded with alginate-encapsulated *P. putida* B2 was used to determine the light response and TCE co-metabolism of *P. putida* B2 when exposed to toluene in immobilized systems. Experimental results showed a rapid bioluminescent response upon the introduction of toluene. Figure 5 shows light response of the reporter strain in the

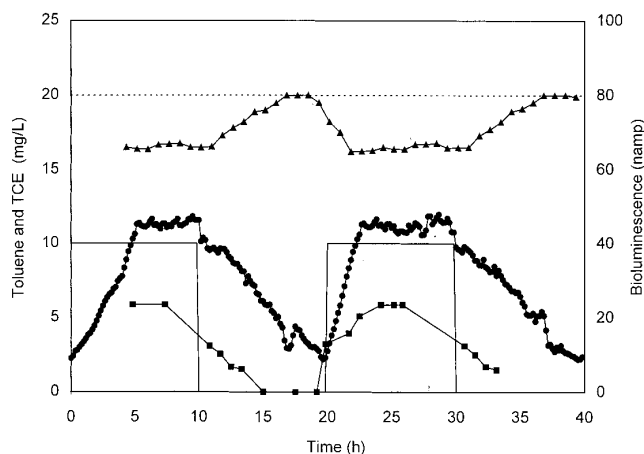


Figure 5 Bioluminescence and co-metabolism of TCE by *P. putida* B2 in response to square wave perturbations of 10 mg L^{-1} toluene in 20-h cycles. Symbols: \bullet bioluminescence, \blacktriangle TCE in effluent, \blacksquare toluene in effluent, ---- TCE in feed, — toluene in feed.

reactor to the change in inlet toluene concentration and removal of TCE. The data show a direct response of bioluminescence with respect to toluene concentration. During the cycle, light emission increased by $16.3 \pm 1.2 \text{ namp h}^{-1}$. The toluene effluent concentration approached zero after the toluene feed was stopped, and the light response in the reactor decreased at a rate of $3.4 \pm 0.8 \text{ namp h}^{-1}$. A direct correlation between bioluminescence and TCE degradation was observed. The maximum light response was $43.4 \pm 6.8 \text{ namp}$. The steady-state TCE effluent concentration when toluene was being introduced into the system was $16.5 \pm 0.2 \text{ mg L}^{-1}$ (20% removal), while the effluent toluene concentration was $5.8 \pm 0.1 \text{ mg L}^{-1}$ (50% removal). This represents a ratio of $1.7 \mu\text{mol}$ toluene degraded μmol^{-1} TCE degraded. While results from the different assay types showed similar response to toluene, the magnitude of bioluminescence cannot be compared due to several differences between experimental conditions (ie sample agitation, cell physiology, light monitoring).

Discussion

Assays of growing cells demonstrated not only a qualitative bioluminescent response to toluene, but a quantitative response as well. There was a linear relationship between bioluminescence and toluene concentration between 0 and 10 mg L^{-1} in assays of growing cells. In addition, the bioluminescent response was proportional to dilutions of a complex environmentally relevant contaminant, jet fuel. However, the magnitude of the bioluminescent response to jet fuel was higher than would be expected if the response was due solely to the toluene in the jet fuel. Work with another bioluminescent strain has recently shown there is a significant bioluminescent response to solvents [8]. It was demonstrated that cells were limited for the aldehyde substrate of the luciferase reaction. It was hypothesized that solvents perturb the cellular membrane, causing intracellular concentrations of fatty acids to increase. Since fatty acids are reduced to the corresponding aldehydes by the *lux* enzymes, increased amounts would negate the aldehyde limitation, causing higher bioluminescence. This solvent effect might explain the observed difference in magnitude of bioluminescence between pure toluene and toluene in a solvent matrix in assays of growing cells.

Typically, in the environment, cells would not be in mid-log phase of growth. Therefore, we examined the bioluminescent properties under resting cell conditions as well. Even in cells with toluene as an intermittent sole carbon source, the bioluminescent response was reproducible for at least 5 days. A more rapid bioluminescent response was observed for cells previously exposed to toluene, but the maximum bioluminescence remained constant.

Immobilized *P. putida* B2 allowed on-line monitoring of degradative activity towards toluene and TCE in a DVR. The system showed a direct correlation between toluene degradation and bioluminescence. Because the *lux* and *tod* operons are under the same promoter control, bioluminescence indicated that the *tod* operon was expressed, and TCE was co-metabolized. Therefore, there was a direct mechanistic correlation between bioluminescence and TCE co-metabolism. In this study, TCE did not appear to induce

the *tod* operon in *P. putida* B2 as was reported for another *P. putida* strain [5]. Figure 5 shows that in the absence of toluene, TCE influent and effluent concentrations were equivalent and there was no bioluminescence increase.

Exposure to TCE and/or its metabolites may be toxic and may affect degradative enzyme activity. However, the intensity of bioluminescence was reproducible in successive perturbations of toluene even in the presence of TCE (Figure 5). These data showed the *tod-lux* reporter provided an on-line measurement of *tod* gene expression, and also provided an indication of potential toxic effects due to continuous TCE exposure. At 20 mg L⁻¹ TCE, there did not appear to be any toxic effects. These studies demonstrated that there is a distinct and reproducible response to toluene under a variety of physiological conditions (growing and resting free cells and immobilized cells). This is the first step in developing this bioreporter technology.

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