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Construction and Characterization of *Escherichia coli* Whole-Cell Biosensors for Toluene and Related Compounds

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Abstract The XylR regulatory protein is a transcriptional activator from the TOL plasmid of *Pseudomonas putida mt-2* that is involved in the toluene and benzene degradation pathway. Here we describe the construction and laboratory characterization of recombinant biosensors (pGLPX plasmids) based on XylR and its cognate promoter (Pu). In the pGLPX plasmid, the reporter *luc* gene is under the control of the Pu promoter. We evaluated the ability of two distinct nucleotide sequences to function as SD elements and improve sensitivity of bioreporting. We also evaluated the effect of introducing the T_2rrnβ terminator on the specificity of the construct. *E. coli* transformed with pGLPX plasmids were used to sense toluene and its derivatives. The pattern of induction was different for each derivative. In general, more luciferase activity was induced by toluene and benzene than by TNT and DNT at most tested concentrations. The bioluminescence response of the reporter strains to the nitrotoluenes was significantly stronger at lower concentrations ($\geq$50 μmol) than at higher concentrations. Our results show that the SD sequence (taaggagg) is crucially important for biosensor sensitivity. The presence of the T_2rrnβ terminator in the bioreporter plasmid prevents nonspecific responses and also reduces biosensor sensitivity upon exposure to inducers. These data suggest that pGLPX strains can be used as whole-cell biosensors to detect toluene and related compounds. Further investigation will be required to optimize the application of pGLPX biosensors.

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Introduction

The contamination of soil and water with non-degradable organic and inorganic pollutants is a threat to public health and the environment. Thus, the ability to monitor, survey, and detect such toxic pollutants by sensitive and convenient methods is important. In addition to chemical analytical methods, several types of bacterial biosensors have been engineered to monitor, detect and even measure the level of contamination in environmental samples [18, 20, 28, 35, 37]. Accurate and sensitive bacterial reporting systems for contaminants provide useful alternatives to direct analytical methods because of their low cost, rapid response, reproducibility, and ease of production.

Over the last few decades, a substantial number of reports have described novel recombinant microbial cells that are capable of chemical detection [1, 4, 11, 17, 36]. These biosensors are typically engineered as fusions between specific promoter sequences and a reporter gene under the control of a transcriptional activator. When bacteria take up organic or inorganic contaminants, the reporter gene under control of the target promoter is activated. There is usually a direct correlation between contaminant concentration and reporter gene expression. The level of reporter gene expression is reflected in the activity of the reporter protein, which can be measured with various instruments, including fluorimeters, luminometers, and spectrophotometers [13, 16, 30–32]. Although many different enzymes and proteins can act as reporter signals, the most commonly used are luciferase (luc) (eukaryotic or bacterial) and fluorescent proteins (e.g., green fluorescent

protein, GFP) because of the specificity of their signals and ease of measuring reporter outputs [7, 21, 29].

BTEX compounds (benzene, toluene, ethyl benzene, and xylenes) are environmental pollutants that are persistent, toxic, and carcinogenic [2, 3]. With genetic reporter constructs, the biodetection and bioavailability of this family of chemicals in environmental samples would be feasible [6, 14, 19, 38]. Genetic reporter constructs may also be applicable to the bioreporting and biodetection of 2,4,6-trinitrotoluene (TNT) and 2,4-dinitrotoluene (DNT), two industrial nitrotoluenes found in explosives. The regulatory proteins XylR and Tbut and their corresponding promoters from the toluene degradation pathways in *Pseudomonas putida mt-2* and *Ralstonia picketti PKOl*, respectively, have been used successfully as a basis for BTEX microbial biosensing [14, 24, 31].

In this work, we describe the construction and laboratory characterization of a recombinant biosensor based on the XylR–Pu system (Pu: upper pathway promoter of Xyl gene). This biosensor detects toluene-based compounds using the firefly luciferase gene as a reporter. We investigated the effect of the Shine-Dalgarno (SD) sequences on the sensitivity of the construct as well as the ability of the T_2rrnβ terminator to reduce nonspecific background.

Materials and Methods

Plasmid Construction

The commercially available luc reporter plasmid pGL_2-Basic (Promega, USA) was used as a starting vector. The *xylR* gene, including its promoter (Pr), was PCR amplified from the TOL plasmid of *Pseudomonas putida mt-2* using the forward primer 5′gcgccaacctatggattttaatgtgggctgctt ggt3′ containing a PfiMI site and the reverse primer 5′cgcggatccttttcacacaacctggggcg3′ containing a BamHI site. The Pu fragment, with or without the upstream terminator sequence, was PCR amplified from the same template. The forward primers 5′gaggtaccaaaaggccatccgtca ggatggccttctggaaagcgcgatgaac3′ and 5′atggtaccggaaagcgc gatgaac3′ were used to amplify the Pu fragments with or without the terminator sequence, respectively (underlined nucleotides correspond to the T_2rrnβ terminator sequence). Each of these forward primers carries a KpnI site; the two primers were utilized in separate reactions that also contained the reverse primer 5′tgagctcgactccaggcgtaacg3′ containing a SacI site. In order to insert SD elements and spacer sequences upstream of the *luc* gene, the above regions were fused with the forward primer containing a XhoI site, SD and spacer sequences in the 5′ overhang using PCR amplification. The template was pGL_2-Basic. The oligonucleotides 5′actcgagtaaggaggttggtaaaatggaaga cgcc3′ and 5′actcgagaggaaattggtaaaatggaagacgcc3′ were used as forward primers; each of these primers contains one kind of SD sequence (underlined nucleotides in the primers correspond to SD_1 and SD_2). These sense primers were used in separate reactions with the reverse primer 5′cgccataggttggtcattacaatttggactttccgcc3′ to amplify the target fragments. All PCR reactions were carried out using the high-fidelity *PFU* DNA polymerase (Fermantas–Lithuania). The PCR products were extracted from agarose gels using a purification kit (Macherey-Nagel, Germany) and cloned into pGl_2-Basic. Pu fragments (in two forms, with and without the terminator) were first inserted into the multiple cloning site with KpnI/SacI. The XylR amplicon was then removed from the multiple cloning site with PfmiI/BamHI. To create the pGLPX biosensor plasmids, the amplicon containing (listed from 5′ to 3′) the SD (SD_1 or SD_2), spacer sequence and the *luc* gene was digested with XhoI/PfmiI and cloned into the linearized recombinant plasmids created in step 2. Figure 1 shows a schematic diagram of the cloning of the pGLPX constructs. T_4 DNA ligase (Fermantas, Lithuania) was used for all ligations. The fidelity of amplicons inserted stepwise into the constructs was verified by regional sequencing of intermediate clones. The final constructs were transformed into DH5α cells for luminescence experiments.

Determination of Organic Compound Concentrations in Culture Medium

To prepare saturated solutions of toluene, benzene, TNT, and DNT, an excess amount of each compound was added to 50 ml of distilled water and the solution was sonicated in an ultrasound bath (Transonic Digital, 800 W for 15 min). After centrifugation, the concentration of organic effectors in the supernatants was determined by measuring the u.v. absorption at λ_{max} and calculating from the extinction coefficient for each compound at λ_{max}. These saturated solutions were used as stock solutions and were added to cell suspensions in appropriate amounts.

Induction Experiments and Luminescence Assay

A single colony of DH5α cells harboring the pGLPX plasmid under study was grown in LB medium containing 100 μmol ampicillin at 37°C. Once the cells had reached an optical density at 600 nm (OD_{600}) of 0.2 (this typically took 7–8 h), the cultivated cells were distributed into tubes and exposed to known various concentrations of effectors. All tubes were wrapped with parafilm during incubation to prevent possible volatilization.

The cultures were incubated at 37°C with agitation at 130 rpm. Cell culture samples were taken at 30, 60, and 90 min after induction and were used immediately for

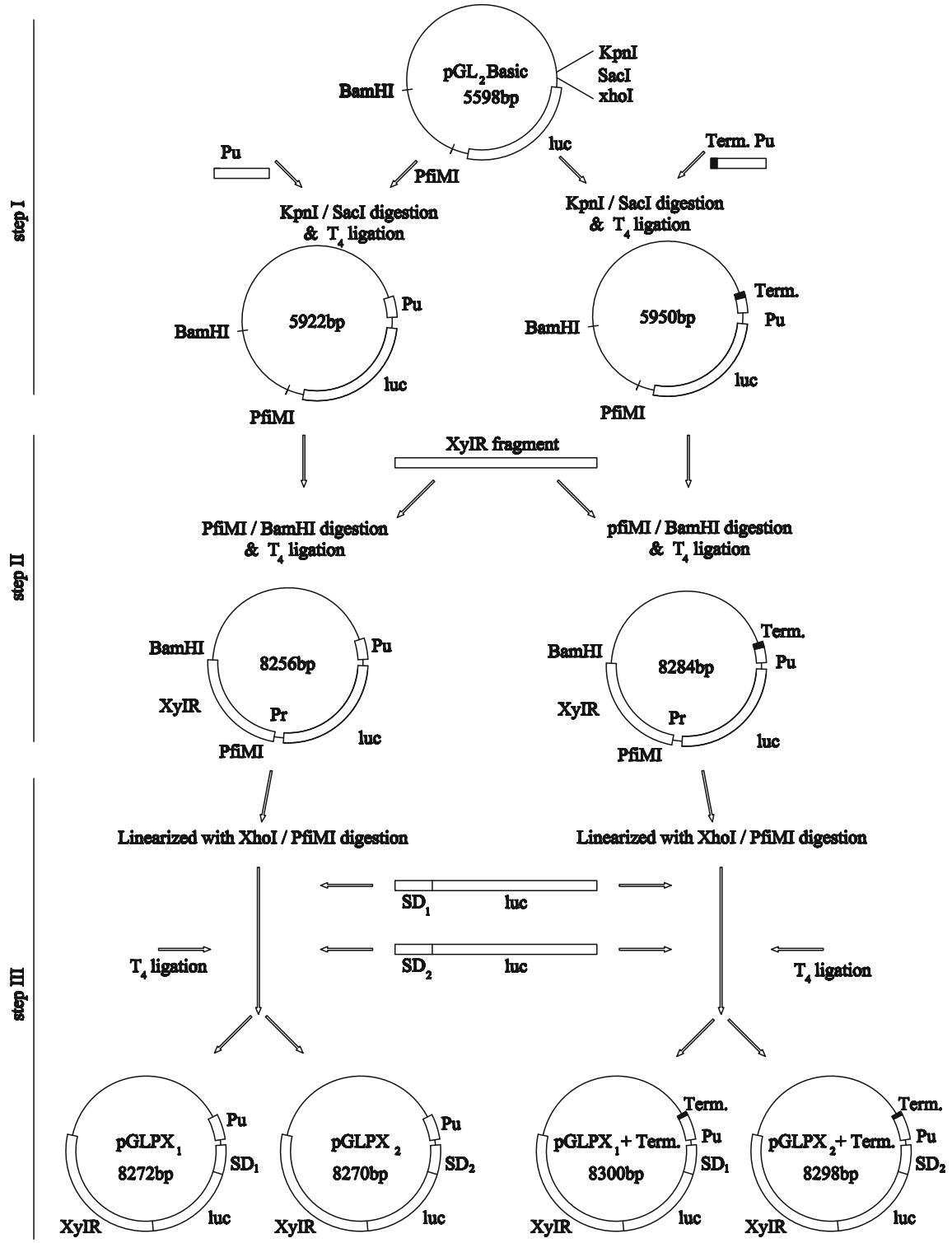

Fig. 1 Schematic diagram of the construction of the pGLPX biosensor plasmids. The diagram is not drawn to scale. *Abbreviations*: *SD* Shine-Dalgarno, *Term.* T_2rrnβ transcriptional terminator, *luc* luciferase

bioluminescence assays. The luminescence measurement was performed directly on culture samples (without lysis) using a "Berthold Orion Microplate Luminometer." In this system, 0.5 mmol (in 50 µl) of D-luciferin (Sigma, USA) was added automatically to each well of a 96-well microplate containing 200 µl of induced cell suspension.

Luminescence was read 5 s after substrate addition. Bioluminescence was expressed as relative light units

(RLU). Three repeats of each sample were measured. Several negative controls (untransformed DH5α cells, cells induced with various concentrations of nitroaromatic compounds, and DH5α cells harboring the corresponding pGLPX plasmid but without induction) were also included.

Statistical Analysis

Each experiment was performed at least three times. The Mann–Whitney *U*-Test was used to compare each treatment condition with the control. All statistical analyses were performed using SPSS Version 12.0 Software (SPSS, Inc., Chicago, Illinois). The significance was set to $P = 0.05$.

Results

Construction of pGLPX-Based Plasmids

A schematic diagram of the construction and key features of pGLPX plasmids is shown in Fig. 1. The Pu region of the TOL plasmid, including the upstream regulatory sequences, the transcription initiation site, and 90 bp of the *xylA* gene (Toluene Oxygenase), is located 41 bp upstream of the *luc* gene at the SacI site [9]. In a subset of clones, the T_2rrnβ terminator (28 bp) is placed in-frame and upstream of the Pu fragment in order to minimize background expression of the *luc* gene [23]. The ribosome-binding site consists of the SD_1 or SD_2 followed by an A-rich translational spacer positioned upstream of the *luc* gene [5]. The *xylR* structural gene (2335 bp), including its cognate promoter (Pr) and autorepressor region upstream of the *xylR* open reading frame, are placed downstream of the *luc* gene in the same direction [9]. The constructed toluene biosensor plasmids were named $pGLPX_1$ and $pGLPX_2$; these contain SD_1 and SD_2, respectively.

Bioluminescent Responses to Nitroaromatic Compounds

We defined the substrate specificity and sensitivity of the XylR–Pu system in the new pGLPX recombinant plasmids by testing the effects of toluene and its structural analogs, including benzene, TNT, and DNT, on reporter expression.

DH5α cells harboring $pGLPX_1$ or $pGLPX_1$+ term (similar but with the terminator) were found to produce consistent luminescence signals in response to toluene and its analogs. The relationship between luciferase expression (luminescence activity), time of treatment and concentration of each analyte was studied.

Figure 2 shows the bioluminescence activity of cells harboring $pGLPX_1$ and exposed to different concentrations

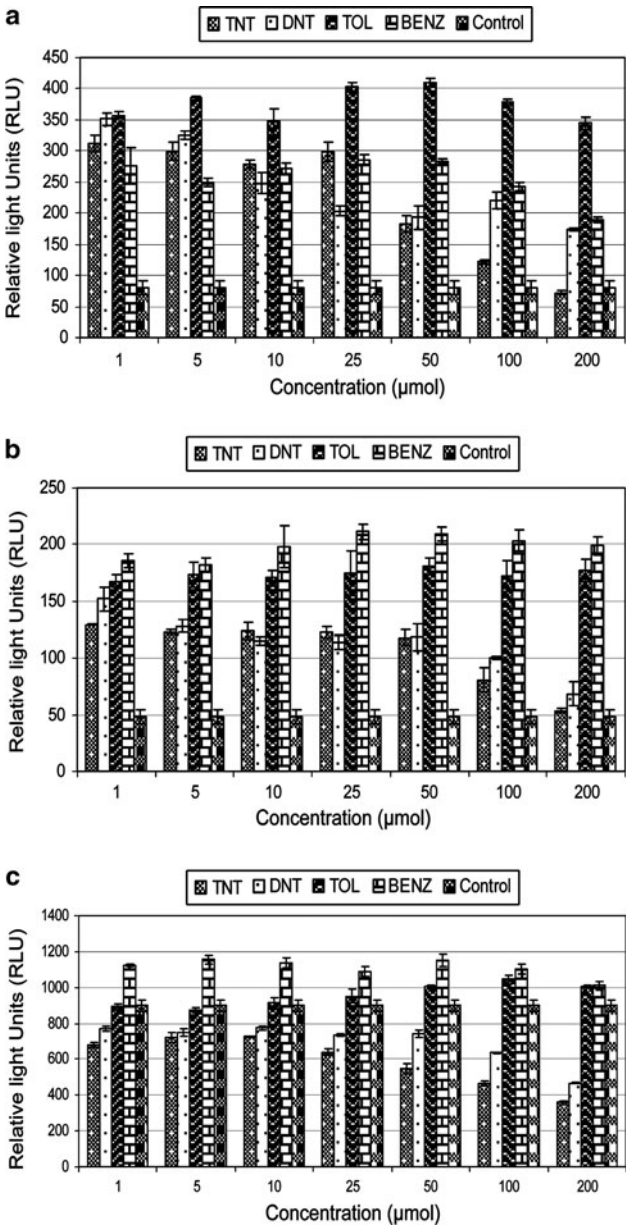

Fig. 2 Comparison of the bioluminescence activities of the $pGLPX_1$ strain in the presence of 1–200 μmol of TNT, DNT, toluene, and benzene. Cells were grown to an OD_{600} of 0.2 and exposed to various compounds for **a** 30 min, **b** 60 min, and **c** 90 min. The control represents the basal activity of *luc* in cells grown under the same conditions without effectors. Bioluminescence is expressed as relative light units (RLU). The data are the average of three independent experiments

of chemicals (from 1 to 200 μmol) measured at different post-exposure times (30, 60, and 90 min). The results show that toluene and benzene are the best inducers of the tested chemicals, particularly after 30 and 60 min. The $pGLPX_1$ strain responded significantly more strongly to these aromatic compounds than to the other analogs at almost all tested concentrations ($P < 0.05$). In particular, the response to toluene after 30 min was found to be

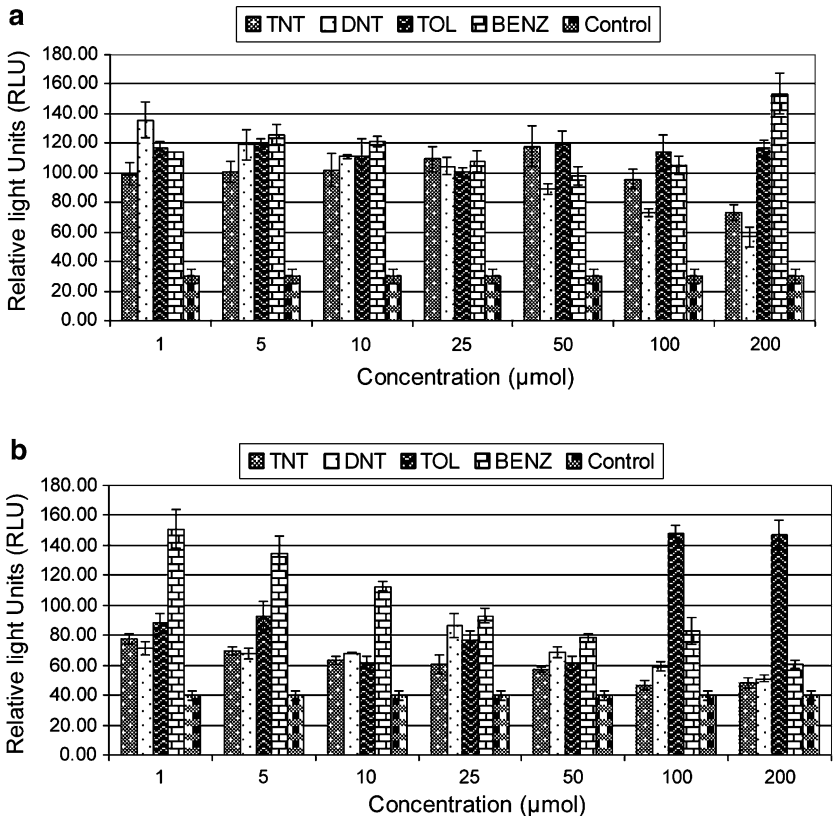

Fig. 3 Comparison of bioluminescence activities of the "pGLPX$_1$+ term" strain in the presence of 1–200 µmol of TNT, DNT, toluene, and benzene. Cells were grown to an OD_{600} of 0.2 and exposed to various compounds for **a** 60 min and **b** 90 min. The control represents the basal activity of *luc* in cells grown under the same conditions without the addition of effectors. Bioluminescence is expressed as relative light units (RLU). The data are the average of three independent experiments

significantly higher (≥fourfold) than background at all concentrations. After 60 min of induction, the bioluminescence responses to toluene and benzene were still remarkable and were higher than the responses to other analogs; however, the background level is also higher at 60 min than at 30 min. The nonspecific (background) response increases even more by 90 min. In general, the bioluminescence response of the pGLPX$_1$ strain was found to be significantly greater at lower (≤50 µmol) than at higher concentrations of TNT and DNT, perhaps because higher concentrations are toxic. As demonstrated in Fig. 2, the pattern remained the same at post-exposure times of 30, 60, and 90 min.

DH5α cells harboring pGLPX$_1$+ term, which possesses the transcriptional terminator, also produce luminescence in response to toluene-like compounds. Although the luminescence intensity of these cells is not significantly altered during a 30 min exposure to effectors, by 60 min significant responses can be observed for all chemicals in the tested concentration range ($P < 0.05$) (Fig. 3a). The response after 90 min of incubation was acceptable only for toluene and benzene at some concentrations. The pGLPX$_1$+ term strain also exhibits weaker responses to higher concentrations of TNT and DNT than to lower concentrations (Fig. 3b).

DH5α cells harboring pGLPX$_2$ or pGLPX$_2$+ term plasmids responded poorly to toluene and its analogs. In most cases, there were no statistically significant changes in the luminescence activity of exposed cells compared to controls (each, $P > 0.05$; data not shown). These results indicate that, in pGLPX, SD$_1$ is more efficient than SD$_2$ at stimulating translational initiation of the reporter gene and that the presence of SD$_1$ leads to higher sensitivity for the detection of toluene-like compounds.

Discussion

Several recombinant microbial biosensors for toluene and related compounds that use the XylR regulatory gene of the TOL plasmid have been described. In this system, toluene or related compounds activate the XylR regulatory protein that positively controls the Pu promoter, resulting in the production of bioluminescence from the Pu-reporter fusion gene [14, 15, 19, 38]. We were inspired by the molecular design used by Willardson et al. [38] to construct pGLTUR. We constructed a cluster of recombinant plasmids and used them to determine whether the addition of genetic regulatory elements would improve biosensor sensitivity and specificity. We measured how the recombinant *E. coli* strains perform upon exposure of intact, whole cells to inducers and determined the behavior of the recombinant biosensors upon exposure to DNT and TNT.

We tested the ability of two nucleotide SD region sequences, SD_1 (taaggagg) and SD_2 (aggaaa), to induce bioluminescence [27]. In both cases, the same effective spacing between the SD site and the start codon of the *luc* gene was used [5, 27]. We observed that the SD_1 sequence is far more effective than SD_2 in inducing reporter gene expression from pGLPX. When cells were exposed to various concentrations of toluene and related compounds, the luminescence of strains carrying SD_2 as the ribosome-binding site was found to be very weak. SD_1 may be more effective in forming the translation initiation complex when mRNA transcripts of the reporter gene are undergoing translation.

The presence of an efficient transcription terminator upstream of the promoter that drives expression of the reporter gene in the pGLPX recombinant plasmids minimizes background transcription [22]. This effect has been overlooked in some studies [12, 19, 25]. However, we inserted the T_2 transcriptional terminator from the *rrnβ* gene termination region of *E. coli* upstream of the Pu promoter to measure how much it can minimize background signal. The T_2 transcriptional terminator sequence has been shown to be an efficient terminator even when present in isolated form [23]. The luminescence results obtained demonstrate that the presence of the T_2rrn*β* terminator in pGLPX reduces basal activity in the absence of inducers but that it also moderately reduces the intensity of the response to inducers. These results indicate the importance of the choice of genetic elements such as SD and terminator sequences in optimizing the specificity and sensitivity of bioreporter plasmids.

The firefly *luc* gene offers many advantages over the bacterial luciferase gene; in addition, the firefly *luc* reporter gene already exists in a starting vector. We therefore used this gene to construct the pGLPX plasmids [21, 26, 29]. Because D-luciferin has extremely poor permeability through the *E. coli* membrane, *E. coli* cells transformed with bioreporter plasmids have often been treated with EDTA or lysis buffer in order to produce sufficient luminescence [14, 34, 38]. Because this process is time-consuming and troublesome for the application of a convenient sensing system, we chose to evaluate the activity of pGLPX bioreporters in intact, whole cells. Our data show that the response to toluene and other effectors is complete after 30 or 60 min induction periods for $pGLPX_1$ and $pGLPX_1$+ term strains, respectively. For both strains, extending the exposure time fails to increase the sensitivity; however, it does increase the background level in the absence of inducers. These strains could perfectly sense toluene and benzene in the tested concentration range of 1–200 μM, although each derivative was found to have different effects. Previously developed bioreporters that use the XylR–Pu system have detection limits for toluene as low as 7.5 μmol [19] and between 10 and 20 μmol [38]. The $pGLPX_1$ and $pGLPX_1$+ term strains have detection limits for toluene as low as 1 μmol when intact cells are used in the assays. This improvement in sensitivity results from the use of appropriate genetic regulatory elements in the bioreporter construct. The use of such molecular regulators thus appears to provide a strong advantage for the design of promoter-based plasmids to detect or bioassay chemical contamination.

We found that in cells containing pGLPX recombinant plasmids, higher levels of bioluminescence are induced by toluene and benzene than by TNT and DNT. The bioluminescence response decreases with higher concentrations of the latter, perhaps due to the intrinsic toxicity of these chemicals. It should also be kept in mind that nitrotoluenes are not natural XylR effectors. The weaker bioluminescence response of pGLPX strains to TNT and DNT may reflect low binding affinities of TNT and DNT for the inactive form of the XylR protein. Molecules that bind with a relatively low affinity to the XylR binding pocket have been suggested to induce a partially active conformation of the XylR [38]. Furthermore, no naturally existing bacterial transcription factor has been shown to respond to TNT and/or to DNT. Recently, Garmendia et al. [8] introduced a laboratory-derived XylR mutant that shows high specificity for and sensitivity to nitrotoluenes [8]. Use of this mutant instead of wild-type XylR in the pGLPX strains would likely result in more specific bioreporting of these chemicals.

Aside from factors directly related to XylR protein function, several additional factors may affect the sensitivities and induction coefficients of biosensors; these include host strain, medium composition, growth phase of the harvested bacteria, and amount of bacteria per measurement [10, 33]. It may be possible to improve the performance of pGLPX biosensors by individually optimizing the above factors.

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