Chapter 17

Engineering Whole-Cell Biosensors with No Antibiotic Markers for Monitoring Aromatic Compounds in the Environment

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Abstract

A cornerstone of Synthetic Biology is the engineering of gene regulatory networks. Construction of such biological circuits has been used not only to elucidate the dynamics of gene expression but also for designing whole-cell biosensors that translate environmental signals into quantifiable outputs. To this end, distinct components of given regulatory systems are rationally rewired in a way that translates an external stimulus (for instance, the presence of one chemical species) into a measurable readout typically fluorescence or luminescence. Various biosensors for BTEX (a mixture of benzene, toluene, ethylbenzene and xylenes) are based on XylR, the main transcriptional regulator of the TOL pathway of *Pseudomonas putida* mt-2. In the presence of its natural effectors (e.g., *m*-xylene, toluene or 3-methylbenzylalcohol), XylR triggers expression of the *upper* pathway genes by means of the *Pu* promoter. Available biosensors combine the *xylR* gene and a direct fusion between the cognate *Pu* promoter and the *luxCDABE* operon, all components stably integrated in the chromosome of *P. putida*. A versatile development of the same biosensing concept is described, aimed at increasing the sensitivity of the genetic circuit toward XylR inducers. The new platform utilizes mini-transposon vectors tailored for engineering an artificial expression cascade that operates as an amplifier of the signal/response ratio of the biosensor. This strategy was applied to the construction of a strain that carries a transcriptional fusion between the *Pu* promoter and T7 RNA polymerase (which becomes under the control of XylR and its effectors), along with a T7 promoter controlling expression of the *luxCDABE* operon. This simple regulatory architecture produced a dramatic increase of bioluminescence emission in respect to the strain that carries only the direct fusion between the *Pu* promoter and the *luxCDABE* reporter.

Key words: Synthetic biology, Bacterial biosensors, Non-antibiotic selection, Genetic stability, Mini-transposons, Orthogonal circuits.

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1. Introduction

Engineering bacteria with novel regulatory networks to produce singular outputs has been one of the topics most addressed by Synthetic Biology (1–4). Construction and implantation of such biological circuits in bacterial hosts have been used both to elucidate fundamental questions of gene expression control and for designing whole-cell biosensors for physical or chemical signals. One biotechnological application of considerable interest lies in the construction of bacteria for monitoring specific chemicals in soil (5, 6). In the cases where such sensors are intended for extensive release, it is necessary to maintain the functionality of the engineered genetic circuit and the corresponding reporter output in the absence of external selective pressure e.g., antibiotic selection, which are commonplace in laboratory setups. This places specific constraints on the genetic tools utilizable in bacteria destined for environmental release, as most existing whole-cell biosensors have been constructed by assembling the reporter system and the sensor module on plasmids bearing antibiotic resistances (7–9).

The most common organization of an engineered biosensor circuit is the one composed of one transcriptional factor (TF) that responds directly to the primary environmental signal and a cognate promoter fused to a reporter gene with an enzymatic or an optical readout (fluorescence or luminescence). Various biosensors of this sort, aimed at detection of BTEX (a mixture of benzene, toluene, ethylbenzene and xylenes) and other aromatics have been engineered in soil bacteria (e.g., *Pseudomonas putida*) tailored for extensive application to sites suspect of containing this type of pollutants. One of the most elaborated examples of this sort involved the development of a dedicated genetic platform for stable implantation of different transcriptional regulators and reporter genes in a variety target bacterial genomes (10). Besides allowing chromosomal integration of thereby formatted gene fusions, the same platform facilitated deletion of all markers that were first employed for selection and screening of the constructs. This is an important requirement for bacteria destined for environmental release, since deliberate discharge of antibiotic resistance genes is considered hazardous and ultimately not acceptable. The downside of monocopy, chromosomally integrated sensor genetic circuits is that the intensity of the reporter readout is limited by the intrinsic specificity, sensitivity, and capacity (i.e., maximum output) of the primary regulator/promoter pair, which varies to a large extent. In this chapter, we describe a genetic platform which maintains the requirements applicable to whole-cell biosensors for environmental release (stable genomic integration, no antibiotic resistances) while multiplying the output by means of an artificial expression cascade based on the T7 polymerase. As a case study, we describe below the construction

and validation of a BTEX biosensor endowed with a simple genetic device based on the toluene-sensing XylR protein of *P. putida* and its cognate promoter *Pu* that amplifies the emission of luminescence when the engineered bacteria face aromatic compounds of this sort.

1.1. Rationale and Components of the T7 Polymerase-Based Amplifying Cascade

The biosensor-building strategy described in this Chapter attempts the formatting of a host strain (in principle, any Gram-negative bacterium) that acts as a standarized *landing pad* for multiple sensor modules. Moreover, since the genetic platform is devised for chromosomal integration of the signal-sensing device and for elimination of antibiotic resistance markers, the TF/promoter pairs can be introduced into such strain for eventual environmental release. Assembly of such whole-cell biosensor is based on vectors that incorporate functional parts of two different transposition systems (1) Tn*5*, that inserts randomly into the bacterial chromosome of a large variety of bacteria (11) and (2) Tn*7* that preferentially targets distinct *att*Tn*7* sites, an event that occurs at high frequency in optimal genomic sites and in one specific orientation (12, 13). The functional organization of these three vectors is sketched in Fig. 1. The first one is plasmid pTn*5*Tel [*T7*→ *lux*], which is the suicide delivery vector for a mini-Tn*5* transposon that carries the *luxCDABE* operon of *Photorhabdus luminescens* behind a strong P_{T7} promoter that can be activated by the corresponding T7 phage RNA polymerase (see Fig. 1a) in a fashion altogether independent of the housekeeping RNAP of the bacterial host. Insertion of this transposon into the target Gram-negative genome is selected by means of the telurite resistance genes, a nonantibiotic marker that is perfectly admissible for environmental release (14). The second component of the vector system is pTn*5*-*T7pol*, which delivers a second mobile element with the array of functional sequences indicated in Fig. 1b. The organization of this plasmid is basically identical to that of pTn*5*-LacZ (10), excepting that the *lacZ* insert has been replaced by a promoterless T7 RNA polymerase (*T7pol*). As a result, *T7pol* is placed downstream of the optimal site for Tn*7* insertion in *E. coli* (*att*Tn*7*; (15)), which lies adjacent to a kanamycin (Km) resistance gene. Finally, the third element of the genetic platform described herein is pTn*7*-*FRT* (10). This is a mini-Tn*7* delivery plasmid that operates as the cloning vector for any sensor module consisting of the gene for the desired signal-sensing TF and its matching promoter (see Fig. 1c). Insertions of the mini-Tn*7* can be selected with gentamicin (Gm) and the marker later deleted as explained below.

Besides the mobile element indicated in each case, the vectors of Fig. 1 bear the *bla* gene for resistance to beta-lactam antibiotics (*bla*) located outside the transposon ends. This allows discrimination, when the time comes, of true transposition events from mere co-integration of the delivery plasmid with the target genomes. Yet, the critical feature of the platform embodied in these plasmids is the presence in pTn*5*-*T7pol* and pTn*7*-*FRT* of purposefully located

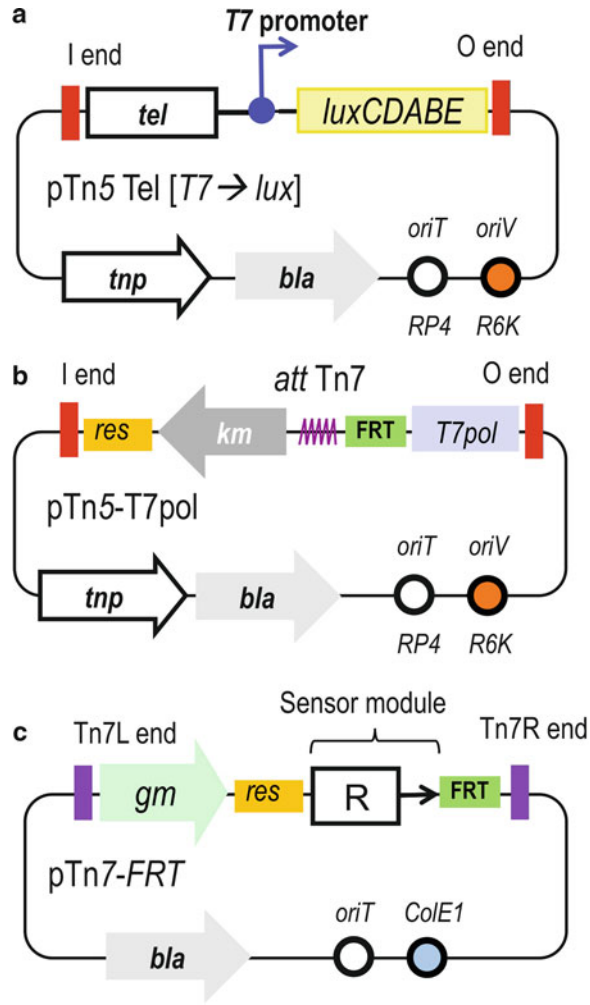

Fig. 1. Structure (not to scale) of the mini-transposon delivery vectors used in this work. (**a**) Structure of mini-Tn*5* transposon suicide delivery plasmid (pTn5 Tel [*T7→ lux*]) bearing nonantibiotic resistance marker (*tel*) and a fusion between the *luxCDABE* operon and the cognate promoter of the RNA polymerase of the bacteriophage T7. (**b**) Structure of mini-Tn*5* transposon suicide delivery plasmid (pTn*5-T7pol*) bearing an optimized *att*Tn*7* sequence, promoterless T7 RNA polymerase gene, *res, FRT* sequences, and Km resistance gene assembled in a conditional replication plasmid. The important elements for both mini-Tn*5* delivery vectors are colored: Tn*5* transposase coding gene *(tnp)*, the origins of replication (*R6K)*, and transference (*RP4*) and resistance gene to beta-lactamic antibiotics (*bla*). (**c**) Structure of mini-Tn*7* transposon delivery plasmid pTn7-*FRT* inserted with the transcriptional regulator and the cognate promoter that form the sensor module. *res, FRT* sequences, the genes that confer antibiotic resistance (*gm* and *bla*), and the origins of replication (*oriT*) and transference (*ColE1*) are colored.

FRT sequences from the yeast *FRT*/FLP site-specific recombination system (16), along with *res* sequences from the multimer resolution system of broad host-range plasmid RP4 (17). As disclosed later in the Chapter, the definite order in which these sequences are arrayed

in each of the vectors allow the eventual deletion of the antibiotic resistance markers and the tractable activation of the biosensor circuit (10).

1.2. Implantation of a Regulatory Cascade for Enhanced Biosensing of BTEX in P. putida Cells Destined for Environmental Release

As a study case, we describe below all steps for engineering a regulatory cascade that amplifies the response of *P. putida* to the aromatics present in BTEX with the judicious use of the three vectors described in Fig. 1. To this end, the overall strategy is briefly described in this section, followed below by complete protocols for each step of the process. The itinerary toward the enhanced and release-able BTEX biosensor starts with the insertion of the *T7→ lux* module of pTn*5*Tel [*T7→ lux*] into the genome of the target strain (*P. putida* KT2440) by selection for resistance to potassium tellurite. This operation enters the output-producing component of the circuit i.e., a P_{T7}→ *luxCDABE* fusion in the genome of the carrier strain. Note that in the absence of the T7 polymerase, transcription of the reporter cannot happen and emission of luminescence is virtually none. One exconjugant from such transposition procedure (see Fig. 2a) was named *P. putida* T7·LUX4 and employed as

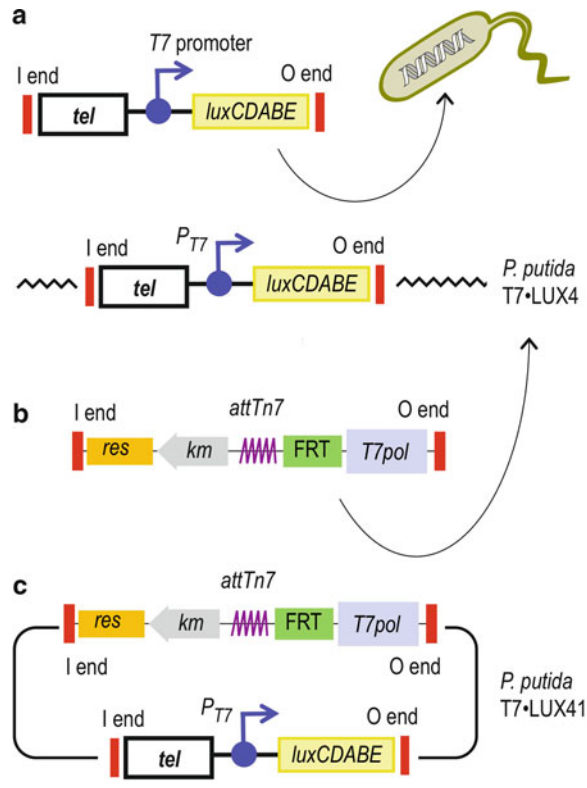

Fig. 2. Construction of the formatted strain that acts as genomic anchor for the sensor circuit in the *prerelease* strain. (**a**) insertion of Tn*5*Tel [*T7→lux*] in the chromosome of *P. putida* generates strain T7·LUX4. (**b**) Insertion of Tn*5-T7pol* in the chromosome of *P. putida* T7·LUX4 produces strain T7·LUX41. (**c**) Organization of relevant insertions in *P. putida* T7·LUX41 (not to scale).

recipient of a second transposition round, in which the donor plasmid was pTn*5-T7pol*. Selection of Km^R colonies allows the appearance of clones which besides the previously inserted Tn 5 Tel [*T7→ lux*], have the complex insert sketched in Fig. 2b in their chromosome. One of such clones (named *P. putida* T7·LUX41, see Fig. 2c) thus contains both segments with P_{T7}→ *luxCDABE* and T7pol, but the later lacks any expression signals and therefore luminescence emission remains as low as *P. putida* T7·LUX4. As indicated in Fig. 3a, strain *P. putida* T7·LUX41 becomes the standarized host for further insertion of the core sensor module, consisting of the gene for a transcriptional regulator along with a cognate promoter. In our example, the regulator is encoded by the *xylR* gene of the TOL plasmid, which acts on the *Pu* promoter (18) This module is first cloned in vector pTn*7-FRT* (see Fig. 1c and Note 1), yielding in our case plasmid pTn*7* [*FRT*-BXPu] (10). In this construct (see Fig. 3a), the TF/promoter segment becomes bracketed upstream and downstream by *res* and *FRT* sequences, respectively. This arrangement of functional segments is itself inside a Gm^R mobile element delimited by the L and R termini of Tn*7*. When this transposon is delivered to *P. putida* T7·LUX41, Gm^R/Km^R exconjugants which are sensitive to the beta-lactam antibiotic piperacillin (Pip) must contain the group of sequences of various origins represented in

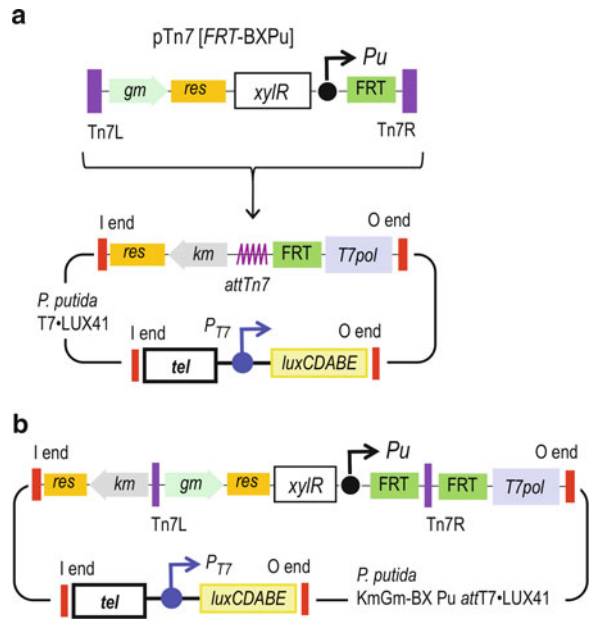

Fig. 3. Insertion of the sensor module into the *landing pad* of the formatted *P. putida* T7·LUX41. (**a**) insertion of mini-Tn*7* carrying *xylR* and its cognate promoter *Pu* into *P. putida* T7·LUX41. (**b**) Genetic structure of the *prerelease* strain carrying Km and Gm markers flanked by *res* sequences, the *xylR* gene and its corresponding promoter *Pu,* and the T7 RNA polymerase gene downstream Tn*7* right end, flanked by *FRT* sequences.

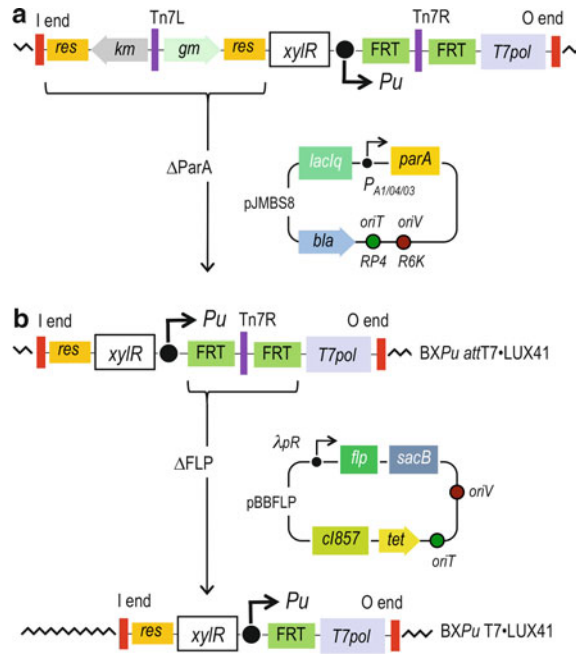

Fig. 4. Enabling the biosensor strain. (**a**) Deletion of antibiotic resistance markers of *P. putida* strain, Km Gm-BX Pu *att*T7·LUX41 by means of site-specific recombination between flanking *res* sequences bought about by transient expression of ParA from pJMBS8 plasmid. The organization of the segment of interest after the deletion is shown in the resulting strain *P. putida* BX Pu *att*T7·LUX41, not to scale. (**b**) Deletion of Tn7 right end placed between *Pu* and the T7 RNA polymerase-coding gene by expression of FLP mediated by pBBFLP plasmid and site-specific recombination between the flanking *FRT* sequences. Note the antibiotic-free, functional organization of the resulting module.

Fig. 3b. This is because the mini-Tn*7* borne by pTn*7*[*FRT*-BXPu] is targeted to the *att*Tn*7* site-engineered upstream of the *T7pol* gene of *P. putida* T7·LUX41.

The resulting strain (*P. putida* Km Gm-BX *Pu att* T7·LUX41; see Fig. 3b) already contains all regulatory and functional elements that are needed for the enhanced BTEX biosensor, but they are at this point in a silent state. The flow of transcription between the *Pu* promoter and the *T7pol* gene is blocked by the transcriptional termination activity of the Tn*7* right end located between them (10). Furthermore, the longer genomic implant of the strain encodes genes for Km and Gm resistance, which were inherited owing to the strategy for selection of each of the insertions. This precludes any possible application e.g., deliberate environmental release. Fortunately, the order and orientation of the business segments of *P. putida* Km Gm-BX *Pu att* T7·LUX41 (see Fig. 4, top) has been arranged to both delete antibiotic resistances, and to fully activate the designed cascade for BTEX detection. Enabling the prerelease strain *P. putida* Km Gm-BX *Pu att* T7·LUX41 (see Fig. 3b) into an active biosensor entails two steps (10). First, in vivo deletion of the

segment of the insert containing the antibiotic resistance markers that is limited by the two *res* sequences (see Fig. 4a, top). This is brought about by the conditional expression of the *parA* gene, encoding the cognate site-specific recombinase of RP4 that is present in the suicidal plasmid pJMBS8 (19) (see Fig 4a). As result of this site-specific recombination, strain *P. putida* Km Gm-BX *Pu att* T7·LUX41 loses the resistance cassettes without affecting the regulatory parts of the engineered signal-amplification cascade, thereby generating strain *P. putida* BX *Pu att* T7·LUX41 (see Fig. 4b). The final step of the enabling process is the removal of the terminator Tn*7*R placed between *Pu* and the promoterless *luxCDABE* reporter. In this case (see Fig. 4b), elimination of the unwanted sequence is affected by the site-specific recombination between flanking *FRT* sequences by expressing the yeast flippase (*flp*, FLP; (16)). The result of this concluding action is a completely functional and environmentally safe biosensor strain named *P. putida* BX *Pu* T7·LUX41 (see Fig. 4b). The plasmid encoding FLP (pBBFLP; see Fig. 4b) can be eliminated by plating the strain on a medium with sucrose, which counterselects the *sacB* gene present in the plasmid (10). The *scar* left after recombination between the promoter of the signal-sensing module (i.e., *Pu*) and the *T7pol* gene does not interfere with expression of the T7 RNA polymerase expression when XylR is activated by BTEX.

Strain *P. putida* BX *Pu* T7·LUX41 hosts all the necessary genetic parts for the amplification cascade sketched in Fig. 5a. In sum, XylR effectors bind this TF, which then activates *Pu* and thus causes expression of the *T7pol* gene. In turn, the produced T7 RNA polymerase triggers the P_{T7} promoter in front of the operon *luxCDABE* operon. As shown in Fig. 5b, this cascade endowed the host strain with a considerable response to the presence in the medium of 3-methylbenzylalcohol (3MBA, a proxy of the BTEX inducers). Depending on the growth phase, light readout was 5–10-fold higher than an equivalent strain bearing a direct fusion *Pu-luxCDABE* (10; see Fig. 5b, c). Needless to say that replacing the sensor module in vector pTn*7-FRT* by any other combination of signal-responsive TF/promoter pairs should suffice to generate similar cascades for any other target compounds or, in general, any environmental stimulus of choice.

2. Materials

2.1. Strains and Plasmids

All the strains and plasmids described in this study are listed in Table 1

2.2. Media and Reagents

1. LB medium: 10 g/L of tryptone, 5.0 g/L of yeast extract and 5.0 g/L of NaCl, dissolve in H_2O and autoclave. For LB-agar plates add 1.5% agar.

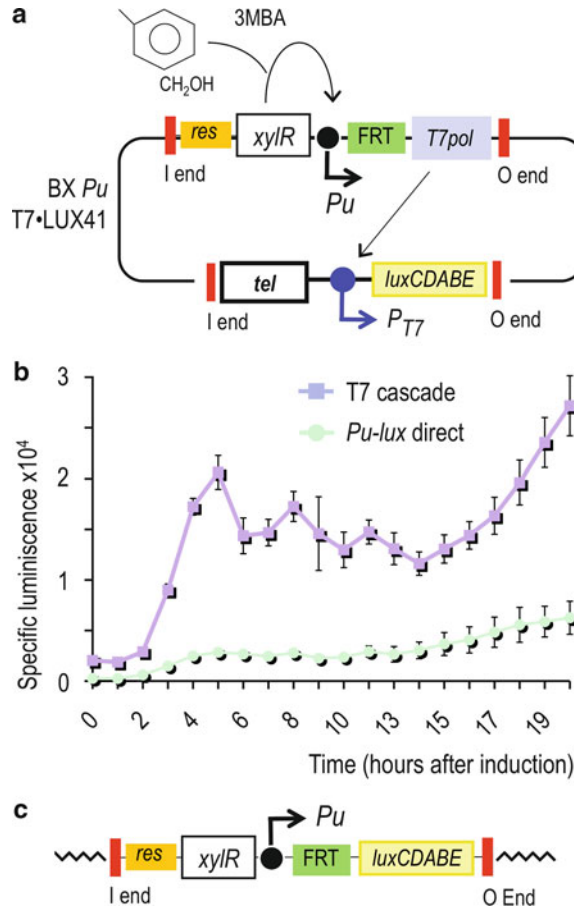

Fig. 5. Response of the BTEX biosensor strain engineered with a transcriptional amplification cascade. (**a**) Sketch of the regulatory circuit borne by *P. putida* strain BX *Pu*T7·LUX41. 3MBA activates XylR, which then triggers the *Pu* promoter and expression of the T7 polymerase. In turn, T7 RNA polymerase activates expression of *luxCDABE*, causing emission of light. (**b**) Luminescence produced by a culture of *P. putida* BX *Pu*T7·LUX41 added with 1 mM 3MBA. In the absence of 3MBA, luminescence is below the detection threshold (not shown). see comparison of the strain engineered with the T7-based cascade and one identical inserted instead with a direct *Pu→luxCDABE* fusion, the organization of which strain (*P. putida* BX *Pu* LUX14; *10*) is drawn in (**c**).

2. Minimal medium M9 preparation (see Note 2 below): First, set a 10× stock solution of M9 salts with 42.5 g Na_2HPO_4-$2H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, and 5 g NH_4Cl, dissolved in 500 mL of H_2O and sterilize; prepare stocks of 1 M $MgSO_4$ and citrate at 20% (as selective carbon source for *Pseudomonas*); also, make a 1.6% agar solution dissolved in H_2O; and autoclave the four components separately. After sterilization, mix components to leave them at the following concentrations: 1× M9 salts, 2 mM $MgSO_4$, 0.2% citrate, and 1.4% agar.
3. Antibiotic concentrations:

Table 1
Strains and plasmids

Strain or plasmid(s)	Relevant characteristics	Reference
Strains		
E. coli CC118 λ*pir*	Δ(*ara-leu*), *araD*, Δ*lac*X174, *galE*, *galK*, *phoA*, *thi1*, *rpsE*, *rpoB*, *argE (Am)*, *recA1*, and lysogenic λ*pir*. Recipient of pTn5 Tel [*T7→lux*], pTn5-T7pol, and pTns1	(26)
E. coli DH5α	Routine host strain for cloning, recipient of pTn7 FRT-BXPu	(22)
E. coli HB101	Sm^r, *hsdR*$^-$*M*$^+$, *pro*, *leu*, *thi*, and *recA*. Host of RK600	(22)
P. putida KT2440	mt-2 cured of plasmid pWW0	(27)
P. putida T7·LUX4	Tel^R, KT2440 with *lux* operon under the control of *T7* promoter	This work
P. putida T7·LUX41	Km^R, *P. putida* T7LUX4 with *T7pol* gene downstream the *att*Tn7 site	This work
P. putida Km Gm-Bx *Pu att*T7·LUX41	Km^R, Gm^R *P. putida* T7·LUX41, and the *xylR/Pu* sensor module inserted upstream the *T7pol* gene	This work
P. putida BX *Pu att*T7·LUX41	*P. putida* Km Gm-Bx Pu *att* T7·LUX41 after the deletion of the antibiotic resistances cassettes	This work
P. putida BX *Pu* T7·LUX41	*P. putida* BxPu *att* T7·LUX41 after the deletion of the Tn7R end	This work
Plasmids		This work
pJMBS8	Ap^R, R6KoriV RP4 *oriT* PA1/04/03::*parA* source of ParA recombinase	This work
RK600	Cm^R; ColE1oriV RK2 *mob*$^+$ *tra*$^+$	(11)
pUC18Not	Ap^R, same as pUC18 but with polylinker flanked by *Not*I sites	(26)
pBBFLP	Tc^R, source of inducible FLP recombinase	(10)
pTn5 Tel[*T7→lux*]	Ap^R Tel^R, pUT mini-Tn5 Tel with a T7 promoter *luxCDABE* operon fusion	This work
pTn5-T7pol	Ap^R Km^R, pTn5 resL Km with *T7pol* gene downstream the *att*Tn7 site	This work
pTns1	Ap^R, RK6 replicon encodes the TnsABC + D specific transposition pathway	(12)
pTn7 [*FRT*-BX *Pu*]	Ap^R Gm^R, pTn7-*FRT* carrying the *xylR/Pu* sensor module	This work

- Ampicillin (Ap) stock: 150 mg/mL in H_2O, filter sterilized. Store at –20°C. For *E. coli* cells use it at a final concentration of 150 μg/mL.
- Kanamycin (Km) stock: 50 mg/mL in H_2O, filter sterilized. Store at –20°C. Use it at a final concentration of 50 μg/mL.
- Chloramphenicol (Cm) stock: 30 mg/mL in ethanol, filter sterilized. Store at –20°C and use it at a final concentration of 30 μg/mL.
- Piperacillin (Pip) stock: 40 mg/mL in H_2O, filter sterilized. Store at –20°C. Use it at a final concentration of 40 μg/mL.
- Tetracycline (Tc) stock: 10 mg/mL with H_2O, filter sterilized. Store at –20°C. Use it at a final concentration of 10 μg/mL.
- Gentamicin (Gm) stock: 10 mg/mL with H_2O, filter sterilized. Store at –20°C. Use it at a final concentration of 10 μg/mL.

4. Tellurite stock: K_2TeO_3 (potassium tellurite hydrate; Aldrich Chemical Co.) dissolved in water at a concentration of 40 mg/mL. The preparation was filtered and kept frozen indefinitely at –20°C. The working concentration of this salt in selective plates was 80 μg/mL.
5. Sucrose solution dissolved at 300 mM in H_2O. Sterilize and maintain at room temperature.
6. 3-methylbenzylalcohol (3MBA) stock solution was dissolved in dimethylsulphoxide (DMSO; Fluka #89890) at a concentration of 1.0 M. Use it at a final concentration of 1 mM. Store at room temperature.

2.3. PCR Primers Used to Verify Targeted Insertion of the pTn7 [*FRT*-BX*Pu*]

In this work we used the following primers (5′→3′) for confirming correct insertion of the *xylR/Pu* sensor module of pTn7 [*FRT*-BX*Pu*] into the att site of the mobile element borne by pTn5-*T7pol*:

- Pu1F: CCCGGGAAAGCGCGATGA, which anneals in the 5′region of the *Pu* promoter of *P. putida*
- T7pol2R: CGGCTTAGGAGGAACTACG, anneals in the T7 RNA polymerase gene present in plasmid pTn*5-T7pol*
- PpuglmS2R: GTGCGTGCCCGTGGTGG, anneals in glutamine synthetase *(glmS)* gene, present in the chromosome of *P. putida*

3. Methods

3.1. DNA Techniques

1. For plasmid preparations we routinely use the Wizard Plus SV Minipreps kit (Promega).
2. PCR-amplified DNA and agarose DNA extractions are commonly purified with the NucleoSpin Extract II kit (MN).

3. In DNA ligations the T4 ligase is employed.
4. PCR: for general PCR reactions prepare first a PCR reaction mix in an Eppendorf tube. The values given below are calculated for a final volume of 25 μL per tube:
   - 5 μL Buffer 5× (provided by the supplier)
   - 1.5 μL $MgCl_2$ 25 mM
   - 1 μL of upstream primer 5 μM
   - 1 μL of downstream primer 5 μM
   - 0.5 μL dNTPs 10 mM
   - 0.3 μL DMSO (specially recommend for organism with a high GC genome content)
   - 0.2 μL Taq Polymerase

   Add 15.5 μL of sterile H_2O into each of the PCR reaction tubes. Pick fresh single colonies directly from a plate and transfer it directly to the PCR reaction tube containing the 15.5 μL of H_2O. Vortex the PCR reaction mixture and distribute 9.5 μL into PCR tubes. Set up the PCR machine with the appropriate Tm (depending on primer composition) and extension time.

3.2. Engineering the Biosensor

P. putida T7·LUX41 strain (see Fig. 2c) was endowed with all the necessary genetic parts that allow construction whole-cell biosensors without antibiotic resistances and with a regulatory cascade that amplifies the output once the sensor module has been introduced. As explained above, this strain was engineered by successive insertions of the mobile elements carried by pTn*5*Tel [*T7*→ *lux*] and pTn*5*-*T7pol* into the genome of *P. putida* KT2440. The corresponding mini-transposons were delivered into recipient cells by three-parental mating procedure (see below). Both plasmids replicate through the *pir*-dependent R6K origin (20; see Fig. 1a, b), so they act as a suicide delivery vectors in any host lacking the π protein. This makes the entire genetic platform to be applicable to a wide variety of gram-negative bacteria so that given strains can be formatted identically to *P. putida* T7·LUX41 for hosting the same regulatory systems. For the sake of illustration, however, we focus below on a detailed description of the methods when applied to *P. putida*.

3.2.1. Construction of Plasmids and Transposons for Circuit Implantation

1. The *BamHI*/*Pst*I 5871 bp segment of pALux4 (10) carrying the *luxCDABE o*peron was cloned into pUC18NotT7 (21) and the corresponding 6071 bp *Not*I fragment (bearing a *PT7* →*luxCDABE* fusion) was passed into pJMT6 (14), producing pTn*5*Tel [*T7*→ *lux*].
2. For assembling pTn5-*T7pol*, the *Xma*I-T7pol-*Pst*I insert from pUC18Not/T7pol (21), carrying the T7 RNA polymerase

gene, was transferred into pBluescript SK- (Stratagene). Then a *BamH*I-T7pol-*Pst*I fragment was cloned into p*attFRT* (10), generating p*attFRT* T7pol. Finally, a *Not*I fragment of this plasmid carrying the T7 RNA polymerase gene was inserted into pUT/mini-Tn*5res*LKm (10), producing pTn5-*T7pol*.

3. pTn*7* [*FRT*-BX*Pu*] was engineered by excising the *Not* I fragment from pBXPu (10), which carries the *xylR/Pu* sensor module, and introducing it into pTn*7*-*FRT* (10).

3.2.2. Suicide Delivery of Mini-Tn5 Derivatives (pTn5Tel [T7→ lux] and pTn5-T7pol)

1. Aerobically grow overnight cultures of:
   - Donor cells: *E. coli* CC118λ*pir* (carrying either pTn*5*Tel [*T7→ lux*] or pTn*5*-*T7pol*) in LB with ampicillin 150 μg/mL and Km 50 μg/mL at 37°C.
   - Helper cells: *E. coli* HB101 (pRK600) in LB with chloramphenicol 30 μg/mL at 37°C.
   - Recipient cells: Grow *P. putida* KT2440 for pTn*5*Tel [*T7→lux*] or *P. putida* T7·LUX4 for pTn5-*T7pol* in LB at 30°C.
2. Mix the three strains in a 1:1:1 ratio (e.g: 100°μL of each strain) in an empty test-tube and complete with 4.7 mL of 10 mM $MgSO_4$.
3. Vortex the mixture and pass through a Millipore filter disc (0.45-μm pore-size, 13-mm diameter) using a 10 mL sterile syringe.
4. Place the filter onto an LB-agar plate and incubate for a maximum of 6 h at 30°C (in this case, this is the optimum temperature for recipient cells).
5. Take the filter from the LB plate with the help of flamed sterile forceps and introduce it into a test-tube with 5.0 mL of 10 mM $MgSO_4$. Vortex to resuspend cells.
6. Plate dilutions (typically 500, 100 and 50 μL from the 5 mL of the resuspended conjugation) on 140 mm diameter Petri dishes with agar-M9 citrate with Km 50 μg/mL or Tel 80 μg/mL (selective media for exconjugant *P. putida* cells inserted with pTn5-*T7pol* or pTn*5*Tel [*T7→lux*], respectively). As negative controls, plate separately *E. coli* CC118λ*pir* (pBAM1), *E. coli* HB101 (pRK600), and the recipient strain on the same medium.
7. Pick exconjugant colonies directly from selective plates with a sterile toothpick and patch on M9 citrate Km 50 μg/mL and Pip 40 μg/mL. This procedure is to make sure that we do not select recipient cells that co-integrated the plasmid instead of undergoing an authentic transposition.
8. Pick a few (5–10) kanamycin resistant and Pip-sensitive colonies. Re-streak single colonies in M9 citrate plus Km 50 μg/mL.

3.2.3. Cloning of Sensor Modules into the Multiple Cloning Site of the pTn7-FRT Vector

Assembly of the sensor module is facilitated by arraying the functional parts (transcriptional regulator and its promoter vector, along with an outward-going cognate promoter) in pUC18Not (11), although it is possible also to directly clone or amplify an existing module as a *Not*I segment (22). One way or the other, the segment is inserted in the *Not*I site of pTn*7-FRT*. In our example, we retrieved the *xylR/Pu* sensor module of pBXPu (10) as a *Not*I fragment, which was then inserted in pTn*7-FRT* (see Fig. 1c) to generate pTn*7* [*FRT*-BX *Pu*].

3.2.4. pTn7-FRT Derivative Delivery by Tetraparental Mating Conjugation

pTn*7-FRT* derivatives carrying any sensor module of interest can then be mobilized to *P. putida* T7·LUX41 strain (see Fig. 3b) by a tetraparental mating in which the Tn*7* transposase is provided in trans with using the helper plasmid pTn*s1* (12) (see Note 2). As before, the following protocol was optimized for *P. putida*:

1. Aerobically grow overnight cultures of:
   - Donor cells: *E. coli* CC118λ*pir* carrying the formatted mini-Tn7 delivery vector that carries the module *xylR/Pu* (pTn*7* [*FRT*-BX *Pu*]) in LB with ampicillin 150 μg/mL and Gm 10 μg/mL at 37°C.
   - Helper cells: HB101 (RK600) in LB with chloramphenicol 30 μg/mL at 37°C.
   - *E. coli* CC118λ*pir* bearing pTns1, the plasmid carrying the Tn*7* transposase genes *tnsABCD*$^+$ in LB with ampicillin 150 μg/mL.
   - Recipient cells: *P. putida* T7·LUX41 in LB at 30°C.
2. Mix the four strains in a 1:1:1:1 ratio (e.g: 100 μL of each strain) in an empty test-tube and complete with 4.7 mL of 10 mM $MgSO_4$.
3. Vortex the mixture and pass through a Millipore filter disk (0.45-μm pore-size, 13-mm diameter) using a 10 mL sterile syringe.
4. Place the filter onto an LB-agar plate and incubate for a maximum of 8 h at 30°C (the optimum temperature for recipient cells).
5. With the help of flamed sterile forceps take the filter from the LB plate and introduce it into a test-tube with 5.0 mL of 10 mM $MgSO_4$ and vortex to resuspend cells.
6. Plate dilutions (in general 500, 100 and 50 μL from 5 mL mix of conjugation) onto M9 citrate with Gm 10 μg/mL (selective media for exconjugant *P. putida* cells) onto 140 mm diameter Petri dishes.
7. Pick colonies directly from transformation plates, with a sterile toothpick and double patch onto M9 citrate Gm 10 μg/mL, Pip 40 μg/mL and on M9 citrate Gm 10 μg/mL. This procedure is to rule out co-integrates (see above).

8. Select gentamicin resistance and Pip-sensitive colonies.
9. Re-streak isolated single colonies in M9 citrate plus Km 50 μg/mL and Gm 10 μg/mL.

3.2.5. Checking the Insertion of the Sensor Module into the Formatted Host Strain by Colony PCR

The mobilization of the pTn*7-FRT* carrying the sensor module into the formatted host bacterium can lead to three different transposition events (1) insertion in the *attTn7* site present in the chromosome of many Gram negatives (12) and placed usually in the terminator of the gene *glmS* (15), (2) insertion into the optimized *att*Tn*7* entered into the chromosome upstream the T7 RNA polymerase by means of pTn*5-T7pol* (see above; see Fig. 2a), and (3) insertion in both loci i.e., the naturally occurring *attTn7* site(s) and the engineered site (see Note 3 below). To discriminate among the three possibilities two PCR reactions are made for each colony analyzed. One uses a sequence corresponding to the promoter of the sensor module as forward primer and T7pol2R that anneals in the position +831 of T7 RNA polymerase gene as reverse primer. The second PCR involves the same forward primer and a sequence annealing in the coding region of the *glmS* as reverse primer (see Note 4 below). The right colonies will be those positives for the first PCR reaction and negatives for the second one. This is because those clones that respond to such a diagnostic PCR have the sensor module inserted upstream the *T7pol* gene, they are amenable for deletion of antibiotic resistance genes and suitable for eventual activation of the regulatory cascade. By using this method we introduced the *xylR/Pu* sensor module into *P. putida* T7·LUX41 strain, generating the strain *P. putida* Km Gm-BX Pu *att*T7 LUX41 (see Fig 3b). The protocol below is optimized for *P. putida* T7·LUX41.

1. Pick single, large colonies from sixteen different gentamicin resistant and Pip-sensitive exconjugants. Re-streak the picked colonies on agar plates with M9 citrate plus Km 50 μg/mL and Gm 10 μg/mL to maintain a backup for each screened colony.
2. Pick fresh single colonies directly from a plate and transfer each colony directly to two different PCR reaction tubes.
3. From each colony perform two different PCR reactions one using the primers Pu1F/T7pol2R and other using Pu1F/PpuglmS2R. The PCR conditions are 5 min at 95°C, 25 cycles of 30 s at 95°C, 30 s at 55°C, 2 min at 72°C, and with a final extra 5 min at 72°C.
4. Analyze the PCR reactions by gel electrophoresis on a 1.5% agarose gel in TAE buffer (run at a constant 60 V). When using the Pu1F/T7pol2R primer pair a single PCR fragment of 1,632 bp should be observed if Tn*7-FRT* is inserted in the *att*Tn7 site present in the *landing pad* provided by the mini-Tn*5*. A single PCR fragment of 837 bp should be observed when

using the Pu1F/PpuglmS2R primer pair if Tn*7*-FRT is inserted in the natural *att*Tn7 site.

5. Perform the following steps using the gentamicin resistant and Pip-sensitive colonies that produce a PCR fragment of 1,632 bp using Pu1F/T7pol2R primer pair, and no amplification in the PCR performed with the primers Pu1F/PpuglmS2R.

3.2.6. Deletion of the Antibiotic Resistance Cassettes

After the insertion of the sensor module into the formatted host strain, antibiotic resistance cassettes are deleted using the ParA/*res* components of the multimer resolution system (*mrs*) of broad host-range plasmid RP4 (19). The location of the sensor module into the engineered *landing pad* leaves the antibiotic resistances genes used during the selection steps (Km and Gm) flanked by *res* sequences of the *mrs* in the appropriate orientation. Expression of the ParA protein mediates recombination between both *res* sequences leading to the loss of the intervening chromosomal DNA fragment. In the case of *P. putida*, expression of ParA is achieved with plasmid pJMSB8 (19). This plasmid can be conjugatively transferred to the target cells, but has a R6K origin of replication (20). This means that pJMSB8 can be passed to the recipient but it cannot be established because the new host lacks the replication π protein (23). Yet, expression of the resolvase during the short residence of pJMSB8 in the *Pseudomonas* recipient is sufficient to effect the predicted excision of the DNA segment without any inheritance of the *parA* gene itself (see Fig. 4a). By using this procedure we generated the *P. putida* BX *Pu att*T7·LUX41 (see Fig. 4b) strain. As before, the protocol below was set up for *P. putida*.

1. Aerobically grow overnight cultures of:
   - Donor cells: *E. coli* CC118λ*pir* carrying pJMSB8, the source of the ParA protein. Grow in LB with ampicillin 150 μg/mL.
   - Helper cells: *E. coli* HB101 (RK600) in LB with chloramphenicol 30 μg/mL at 37°C.
   - Recipient cells: *P. putida* Km Gm-BX Pu *att*T*7*·LUX41 in LB at 30°C.
2. Mix the three strains in a 1:1:1 ratio (e.g: 100 μL of each strain) in an empty test-tube and complete with 4.7 mL of 10 mM $MgSO_4$.
3. Vortex the mixture and pass through a Millipore filter disc (0.45-μm pore-size, 13-mm diameter) using a 10 mL sterile syringe.
4. Place the filter onto an LB-agar plate and incubate for a maximum of 8 h at 30°C (optimum temperature for recipient cells).
5. With the help of flamed sterile forceps, take the filter from the LB plate and introduce it into a test-tube with 5.0 mL of 10 mM $MgSO_4$ and vortex to resuspend cells.
6. Plate dilutions (make serial dilutions from the mix of conjugation to get single colonies) onto M9 citrate plates without antibiotics.

7. Incubate overnight and pick colonies directly from conjugation plates, with a sterile toothpick. Patch the same clone on M9 citrate Gm 10 μg/mL, M9 citrate Km 50 μg/mL, and M9 citrate without any antibiotic.
8. Re-streak clones sensitive to both kanamycin and gentamicin in M9 citrate without antibiotics.

3.2.7. Activation of the Biosensor by Deleting the Tn7R End of the Tn7 Transposon

After deletion of the antibiotic resistance cassettes, the last step to generate the release-able, optimized whole-cell biosensor is the enabling of the reporter system. In order to do this, it is necessary to delete of the Tn*7*R end between the promoter of the sensor module and the *T7pol* gene. This is affected by a site-specific recombination event between the two *FRT* sequences that are placed at both sides of the segment that causes transcriptional termination activity (see Fig. 4b). The yeast *FRT*/FLP site-specific recombination system is known to be functional in a broad host-range of host cells (16). In addition, the *scar* left after recombination is known not to produce polar effects in downstream genes (24). On this basis any DNA segment flanked by properly oriented *FRT* sites can be deleted through the transient expression of the yeast flippase (FLP). The expression of the FLP protein is achieved in this case by introducing into the formatted biosensor strain the *flp*$^+$ plasmid pBBFLP (see Fig. 5a; (10)). This is a mobilizable construct based on the pBBR1MCS series (25), able to replicate in *Pseudomonas* and encoding expression of the yeast *flp* gene under the control of a thermosensitive λ repressor (16). This plasmid is endowed also with the *sacB* marker from pFLP2 (16), thereby its loss can be selected by sucrose selection. The expression of the FLP protein inside the *P. putida* BX Pu *att*T7·LUX41 led us to obtain the fully functional biosensor strain *P. putida* BX Pu T7·LUX41 (see Fig. 4b). The complete process of enabling the biosensor strain includes three steps (1) introduction of the vector pBBFLP, source of FLP, (2) screening of functional strains, and (3) curation of pBBFLP. The corresponding protocol for *P. putida* follows.

3.2.8. Delivery of pBBFLP

1. Aerobically grow overnight cultures of:
   - Donor cells: *E. coli* DH5α (pBBFLP). Grow in LB with Tc 10 mg/mL.
   - Helper cells: *E. coli* HB101 (pRK600). LB with chloramphenicol 30 μg/mL at 37°C.
   - Recipient cells: *P. putida* BX *Pu att*T7·LUX41 in LB at 30°C.
2. Mix the three strains in a 1:1:1 ratio (e.g., 100 μL of each strain) in an empty test-tube and complete with 4.7 mL of 10 mM $MgSO_4$.
3. Vortex the mixture and pass through a Millipore filter disc (0.45-μm pore-size, 13-mm diameter) using a 10 mL sterile syringe.

4. Place the filter onto an LB-agar plate and incubate for a maximum of 6 h at 30°C.
5. Collect the filter from the LB plate, introduce it into a test-tube with 5.0 mL of 10 mM $MgSO_4$, and vortex to resuspend cells.
6. Plate dilutions (100 and 50 μL from the mix of conjugation) on agar plates with M9 citrate, Tc 10 μg/mL and incubate at 37°C (see Note 5 below).

3.2.9. Identification of Functional Strains

1. Pick colonies directly from conjugation plates with a sterile toothpick and streak out on plates with M9 citrate, Tc 10 mg/mL.
2. Pick fresh, well-isolated colonies directly from the plate and transfer them directly to different PCR reaction tubes.
3. From each colony, perform a PCR reaction using the pair of primers described previously (Pu1F and T7pol2R). The PCR conditions are: 5 min at 95°C, 25 cycles of 30s at 95°C, 30s at 55°C, 2 min at 72°C, and with a final extra 5 min at 72°C.
4. Analyze the PCR products by gel electrophoresis on a 1.5% agarose gel in TAE buffer (run at a constant 60 V). A single PCR fragment of 1195 bp should be observed when using the Pu1F/T7pol2R primer pair in those colonies that have deleted the Tn*7*R sequence because of the recombination between the *FRT* sequences. In the cases where the Tn*7*R sequence has not been deleted, the PCR fragment should be of 1,632 bp. In the successful instances, elimination of the Tn*7*R sequence allows read-through transcription from the *Pu* promoter into the *T7pol* gene (see Fig. 4b). One of the colonies is then taken to the next step of the procedure.

3.2.10. Curation of pBBFLP

Since pBBFLP contains the *sacB* counterselection marker that confers sucrose sensitivity to Gram-negative bacteria, the spontaneous loss of the plasmid can be selected by plating in a medium with sucrose. Almost all sucrose-resistant colonies may have been cured of pBBFLP, although in some few cases sucrose resistance may be attributable to *sacB* point mutations. These two possibilities can be distinguished by verifying if the sucrose-tolerant colonies are still resistant to Tc (the selection marker of pBBFLP).

1. Streak out the colony from the previous procedure (3.2.9) on agar-M9 citrate.
2. Grow the colony on the same liquid medium supplemented with 5% sucrose, followed by an overnight incubation at 30°C.
3. Plate dilutions of the overnight culture on a M9 citrate 5% sucrose plate.
4. Pick colonies with a sterile toothpick directly from the M9 citrate 5% sucrose plate and patch on plates either with M9 citrate 5% sucrose, Tc 10 mg/mL or M9 citrate 5% sucrose plate.

5. Select a sucrose-resistant and tetracyclin-sensitive colony.
6. Re-streak isolated single colony on agar-M9 citrate. The resulting clone is the concluding biosensor strain that were after.

3.3. Measuring Bioluminescence Production

Once the biosensor strain is in place, the next step is to verify its response toward aromatic compounds. Although the ultimate target in our case is BTEX, we can use 3MBA as proxy of the aromatic mixture, because this chemical is also a good XylR inducer. Production of bioluminescence can be recorded with different devices for detection of light emission. The procedure to capture the output of *P. putida* BX *Pu* T7·LUX41 when exposed to 3 MB is the following:

1. Grow the biosensor *P. putida* strain overnight in 2 mL of LB medium at 30°C, set in 10 mL tubes.
2. Dilute to an OD_{600} of 0.05 in 100 mL flasks and grow to $OD_{600} = 1.0$, at which point 3MBA is added at a concentration of 1.0 mM.
3. Place three replicates of 200 μL aliquots of the cultures (induced and not induced) in 96-well plates (NUNC).
4. Record the growth (OD) and light emission along with the time in a Victor II 1420 Multilabel Counter (Perkin Elmer). The bioluminescence output is calculated as the ratio between total light emission (in arbitrary units) divided by the optical density of the culture (OD_{600}).

4. Notes

1. pTn7-*FRT* replication is based on a ColE1 origin of replication; this fact implies that it cannot be used in bacteria where it can replicate. An alternative is to enter the sensor modules in the previously described vector pCAC5 (10), which replicates through a *pir*-dependent R6K origin (20). This makes the plasmid to be a suicide delivery vector in any host lacking the π protein.
2. Instead of the M9 citrate medium used here for *Pseudomonas*, other bacterial species may require different selective media that allows only the growth of recipient exconjugants.
3. If a high frequency of double insertions of the pTn7-*FRT* carrying the sensor module is found, it is possible to decrease the concentration of the Gm in the media to enrich for exconjugants with only one insertion.
4. For verification of insertions of the mini-Tn7 transposons derived from pTn7-*FRT*, it is recommended to employ a primer matching

a *glmS* coding region somewhat distant from the minimal sequence required for Tn*7* insertion *att*Tn*7* (15).

5. FLP production from pBBFLP is under the control of the temperature-labile λ repressor (16). It is thus recommended to plate the exconjugants after the introduction of pBBFLP at 37°C (yet, complete excision is often observed at 30°C as well).

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