Biosensors

Technical Field

The invention relates to the use of biosensors for detecting environmental signals based on the measurement of genetic responses of cells to those signals.

Background Art

The standard approach for biosensor measurements "based on genetic responses" is to attach reporter genes to the relevant promoters and to measure the signal (which consists of the activity of the enzyme encoded by the reporter gene) in response to the analyte of interest. The first publication dealing with the use of the bacterial luciferase operon to achieve such measurements appeared in 1984 (Baldwin *et al.*, 1984). Since that time many more publications detailing the use of this reporter to probe the activity of promoters have appeared. Today there are probably several thousand publications on this subject. Most of these publications rely on the use of luxAB fusions or of entire lux cassettes. The more restricted goal of measuring the concentration of pollutants with such constructs was first proposed and demonstrated by Gary Sayler's group at the University of Tennessee in the USA (King *et al.*, 1990). Burlage & Kuo (1994) recently reviewed the application of such biosensors with respect to environmental monitoring applications.

Practically all of the constructs described utilise either the entire lux operon for activity measurement or the luciferase part of it (lux AB, in this case the activity is measured by external addition of the aldehyde substrate). The major disadvantage of using the entire lux operon is that production of the enzyme responsible for generating the substrate of luciferase (the fatty acid reductase encoded by lux CDE) occurs simultaneously with the synthesis of luciferase. It is therefore probable that the amount of substrate produced by the cell will be insufficient for saturation of luciferase. The present inventors have introduced modifications into constructs suitable for biosensors in an attempt to address this disadvantage thereby allowing maximal light output as soon as luciferase is synthesised.

Disclosure of the Invention

In a first aspect, the present invention consists in a genetic construct for use in a biosensor comprising:

(a) a first nucleic acid molecule including a sequence encoding a reporter molecule having a detectable activity; and

(b) a second nucleic acid molecule including a sequence encoding an enzyme which produces a substrate for the reporter molecule, the first sequence being under the control of a first inducible promoter and the second sequence being under the control of a second inducible promoter.

In a preferred embodiment, the first nucleic acid molecule encodes bacterial luciferase Lux AB or a functional equivalent thereof and the second nucleic acid molecule encodes Lux CDE enzyme fatty acid reductase or a functional equivalent thereof. These genes may be obtained from any of the *lux* operons of bioluminescent microorganisms, most of which belong to the genera *Vibrio*, *Xenorhabdus*, *Photorhabdus* and *Photobacterium* (Meighen, 1994). The detectible activity in this system is the generation of light.

It will be appreciated that any inducible promoters will be suitable for the present invention. Examples of some promoters that are suitable are listed in Appendix 2. This list, however, is not an exhaustive list but is provided to demonstrate the large number of possible promoters. The choice of the first promoter is often dependent on the environmental signal to which the biosensor is adapted to react. In particular, when the biosensor is used to detect xylene, the *Pu* promoter is especially suitable as the first promoter.

A further preferred embodiment of the first aspect of the present invention is the genetic construct adapted for the detection of xylene set out in Fig. 14.

The distinct advantage of the genetic construct of the present invention is that when in a cell, expression of the enzyme can be induced separately from the expression of the reporter molecule. This allows the cell to be loaded with substrate produced by the enzyme. The substrate is immediately available for use by the reporter molecule when it is produced, thereby giving a quick response to the signal detected. When the reporter molecule is made in response to an environmental signal, it can react maximally to give a detectable activity. Preferably the first promoter is inducible by exposure to an environmental signal to be tested. Induction can

be achieved directly by the signal or indirectly by activating one or more separate pathways in the cell containing the construct.

The genetic construct can include further nucleic acid molecules encoding auxiliary element or elements required for activation of the reporter molecule via its promoter. Examples include regulatory genes or nucleic acid molecules expressing "second message" molecules to activate the first promoter.

The effector specificities of existing promoters which depend on an indirect activation pathway by regulatory genes can be altered by mutation of the signal binding site of the regulatory protein (Ramos *et al.*, Proc. Natl. Acad. Sci. USA, 83, 8467-8471, 1986). New promoters can be identified using promoterless promoter-probe vectors based on transposons such as those described by deLorenzo *et al.* (deLorenzo, V.; Herrero, M.; Jakubzik, U. & Timmis, K. N. J. Bact., 172, 6568-6572, 1990) or Sohaskey *et al.* (Sohaskey, C. D.; Im, H. & Schauer, A. T., J. Bact., 1674, 367-376, 1992).

In a second aspect, the present invention consists in a biosensor for measuring an environmental signal comprising a cell including the genetic construct of the first aspect of the present invention and a means for measuring the activity of the reporter molecule in the cell when the cell has been exposed to the environmental signal.

The cell can be any cell including bacterial, yeast, fungal and other plant cells and animal. Preferably the cell is a bacterial cell. The environmental signal can be pollutants, toxins, temperature, irradiation, biological, and chemical.

When the bacterial luciferase system is used, the basic genetic units of the sensor are the second nucleic acid molecule encoding the production of the fatty acid reductase (unit 2), the first nucleic acid molecule encoding the reporter element containing the luciferase genes and the first promoter (unit 1) as well as in some instances a third unit which would supply auxiliary elements for activation of the sensor element such as regulatory genes (unit 3). In commercial sensors all elements would be ideally inserted in the chromosome of the host organism. Alternatively, all units may be placed on plasmids, or a combination of chromosomally inserted elements and plasmid-borne constructs may be used. For example, where three units are used, unit 2 and unit 3 may be inserted in the chromosome whilst unit 1 is located on a plasmid.

A functional biosensor unit using the bacterial luciferase system consists of a device where the sample to be tested is contacted with biosensor cells which include a genetic construct according to the first aspect of the present invention (in a cuvette for example) and an instrument capable of measuring the light output of the cells. Any light measuring device would in principle be applicable, but the most appropriate systems are photomultipliers, charge coupled devices, luminometers, photometers, fiber-optic cables or liquid scintillation counters. These systems can be portable or laboratory based, they can be adapted for single analysis or high throughput devices for multiple analyses. A survey of more than 90 commercially available systems suitable for the present invention has been published by Stanley in J. Bioluminesc. Chemiluminescence, 7, 77-108, 1992 and regular updates have appeared since.

Analytes can be measured by the biosensor when adsorbed to surfaces, dissolved in liquid media or when present in the gasphase. Condition for measurement is that sufficient moisture be available to ensure the function of the biosensor cells which include a genetic construct. Ideally, samples are diluted with a buffer of appropriate composition prior to analysis and incubated in this solution. Alternatively, analytes of interest may be separated from the sample prior to analysis using means such as solvents or selectively permeable barriers such as membranes. Analytes may also be concentrated prior to analysis by, for example, passing a gas sample through a liquid or through a suitable solid adsorbent.

The biosensors can be used to detect organic or inorganic chemical or environmental signal which elicits a genetic response in an organism. Microbial systems are targeted primarily, but mammalian, plant or fungal promoters can also be used. In addition, the biosensors can be used to detect physiological responses such as starvation, toxicity or sporulation by inserting the luciferase genes behind promoters suitable for physiological measurements. The biosensors can be used to detect pollutants attached to particles, dissolved in aquatic samples or dispersed in the gas phase. The measurements usually need, however, to be performed in the liquid phase.

In a third aspect, the present invention consists in a method of detecting an environmental signal comprising exposing a biosensor according to the second aspect of the present invention to the signal such that the signal induces the expression of the reporter molecule of the cells in the

biosensor, prior to or during exposure the cells being induced to produce the enzyme such that substrate is formed in the cells, allowing the reporter molecule to react with the pre-formed substrate to form a detectable reaction, and detecting the reaction.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following drawings.

Brief Description of the Drawings

Figure 1 is a diagram of the *lux* operon;

Figure 2 is a diagram of clone designated pLuxAB;

Figure 3 is a diagram of clone designated pLuxCDE;

Figure 4 is a diagram of clone designated pTlux;

Figure 5 is a diagram of the two operons controlling the genes encoding the degradation of toluene the Tol operon;

Figure 6 is a diagram of pXlacR/S;

Figure 7 is a diagram of the features of construct designated pXylR;

Figure 8 is a diagram of the construct designated pTU/M;

Figure 9 is a diagram showing the respective positions of the components in pTLU/M;

Figure 10 is a diagram of a representative of the transformant designated plu-x;

Figure 11 is a diagram of a representative of the transformant designated pLEU;

Figure 12 shows the time course of bioluminescence of the new sensor construct (Leu-lacR) with addition of xylene (full squares) and without addition of xylene (open squares) as well as the corresponding data for the control construct (Lux-lacR) where closed and open circles represent the response in the presence of xylene and in the absence of xylene, respectively;

Figure 13 shows a calibration curve obtained with the new sensor construct (Leu-lacR). The organisms were inoculated from a glycerol stock and grown overnight at 30°C with minimal agitation; and

Figure 14 shows a map pf plasmid pLEU.

Detailed Description of the Invention

All components used in this invention are readily available to the public (except for the different constructs generated specifically for this work).

MATERIALS & METHODS

Culture conditions

Recombinant strains of *Escherichia coli* were routinely cultured aerobically at 37°C in Luria broth (LB) - tryptone 10 g/L, NaCl 10 g/L and yeast extract 5 g/L. Media was solidified by the addition of 15 g/L Kobe agar. Filter sterilised antibiotics were added as required. Ampicillin at 100 mg/ml was used to maintain constructs based on the following vectors: pSP72, pUC18, pTrc 99A and pPL-L; and tetracycline at 10 mg/ml for constructs based on pRK404. The different carbon sources used were glucose (1% w/v) and lactose (1% w/v).

General recombinant DNA procedures

Restriction enzymes and other DNA modifying enzymes were used under the conditions specified by the suppliers. Standard procedures as described by Sambrook *et al* (1989) were used for manipulating and cloning DNA. Isolation of plasmid DNA from *E. coli* was performed according to the method described by Morelle (1989), for rapid screening of plasmid-bearing clones.

Table 1: Description of clones constructed during this study

Constructs	Vector	Comments/description
pxylac-R	pRK404	XylR ORF fused to lacZ promoter
pXylR	pRK404	XylR gene including P_r cloned into the PstI/BamHI site of
	pRK404	
pTer	pSP72	Contains both rrnbT1T2April 96 terminators
pTu	pSP72	Pu cloned into the EcoRI/SmaI site of pTer
pluxAB	pUC18	luxAB genes fused to lacZ promoter
pTLU	pSP72	luxAB genes fused to Pu promoter in pTu
pluxCDE	pTrc 99A	luxCDE genes fused to trc promoter
pluxCDABE	pTrc 99	luxCDABE genes fused to trc promoter
pTluxCDABE	pSP72	luxCDABE genes cloned into pTer
pLu-x	pSP72	luxCDABE genes fused to Pu promoter in pTu
pLEU	pTrc 99A	Pu-luxAB detection cassette cloned into the BamHI/PstI site of pluxCDE

Table 2: Description of clones constructed during this study

Constructs	Vector	Date constructed
pxylac-R	pRK404	available in Aug 1994
pXylR	pRK404	constructed 21.4.96 (page 6, Karen Jury's labbook) confirmed 26.4.96 (page 8, Karen Jury's labbook)
pTer	pSP72	available in Aug 1994
pTu	pSP72	available in Aug 1994
pluxAB	pUC18	constructed 3/7/95 (page 28121, Tony Vancov labbook 1) confirmed 6/7/95 (page 28125, Tony Vancov labbook 1)
pTLU	pSP72	constructed 25/7/95 (page 28141, Tony Vancov labbook 1) confirmed 1/8/95 (page 28146, Tony Vancov labbook 1)
pluxCDE	pTrc 99A	constructed 25/7/95 (page 28141, Tony Vancov labbook 1) confirmed 9/8/95 (page 28153, Tony Vancov labbook 1) confirmed aldehyde producer 10/8/95 (page 28154, Tony Vancov labbook 1)
pluxCDABE	pTrc 99	constructed 13/11/95 (page 9122, Tony Vancov labbook 2) confirmed 17/11/95 (page 9124, Tony Vancov labbook 2)
pTluxCDABE	pSP72	constructed 16/5/96 (page 33417, Tony Vancov labbook 3) confirmed 17/5/96 (page 15, Karen Jury's labbook)

Table 2, continued

Constructs	Vector	Date constructed
pLu-x	pSP72	constructed 21/4/96 confirmed 21/4/96 luciferase assay (page 5, Karen Jury's labbook) confirmed 21/4/96 (gel digest, page 6 Karen Jury's labbook)
pLEU	pTrc 99A	constructed 8/12/95 (page 9142, Tony Vancov labbook 2) confirmed 13/12/95 (page 9143, Tony Vancov labbook 2) confirmed 14/12/95 (plate assay, page 9144, Tony Vancov labbook 2)

Table 3 Recombinant *E. coli* strains

Code	*E. coli* strain	Constructs*	Date obtained
Tlu-lacR	NM522	pTLU + pxylac-R	constructed 7/8/95 (page 29151, Tony Vancov labbook 1) confirmed 10/8/95 (page 28154, Tony Vancov labbook 2)
Leu-lacR	NM522	pLEU + pxylac-R	constructed 16/4/96 (page 33404, Tony Vancov labbook 3) confirmed 20/4/96 (pages 33407 and 33408 Tony Vancov labbook 3)
Leu-R	NM522	pLEU + pXylR	constructed 10/5/96 (clone (i)e in Karen Jury's labbook) confirmed 11/5/96 (luminometer readings and gels, page 13 Karen Jury's labbook)
Lux-LacR	NM522	pLu-x + pxylac-R	constructed 17/5/96 (page 33418, Tony Vancov labbook 3) confirmed 18/5//86 (pages 14-16, Karen Jury's labbook)
Lux-R	NM522	pLu-x + pXylR	constructed 10/5/96 (clone (ii)g in Karen Jury's labbook) confirmed 11/5/96 (luminometer readings and gels, page 13 Karen Jury's labbook)

*note that constructs carrying the XylR regulatory gene (pXylR or pxylac-R) are located on the pRK 404 plasmid whilst the actual light generating elements of the sensor are inserted in the plasmids pSP72 (pLu-x) or pTrc 99A (pLEU).

Bioluminescence Assays - preparation of culture

Unless otherwise stated, *E. coli* clones were inoculated from a fresh agar plate into a flask containing 40 ml of LB media with the appropriate antibiotics and grown aerobically (with shaking) at 37 ^{o}C for approximately 2-3 hr. The culture was harvested at an OD_{600} of approximately 0.8-1.2, washed twice and resuspended in a buffer containing: 100 mM Phosphate buffer pH 7.5, 1/10th diluted LB broth and 0.1% glucose (w/v). In some cases, specified in the text, inducers and/or effectors such as IPTG and m-xylene (1 mM) were added. The optical density of the culture was adjusted to 1.6 and assayed as follows:

Ten ml of cell suspension were poured into in a 100 ml tightly sealed bottle (Schott bottle) which was placed on a magnetic stirrer at room temperature for the duration of the experiment. Sampling was performed at either 20 min or 30 min intervals. An aliquot of 100 ml of culture was removed and the optical density of the culture was measured at 600 nm to measure biomass content. The light production (bioluminescence in Lumi Counts Per Second, LCPS) was measured with a MicroBeta scintillation counter using microtiter plates. Twenty μl of 20 % glucose were added to each well of the micro-titre plate (glucose acts as an electron donor). In each sample, luminescence activity was recorded with and without the addition of 0.5% tetradecyl aldehyde. Specific activity is determined by dividing the LCPS by the OD_{600}.

Aldehyde indicator plates

Mixtures of pararosaniline and bisulfite are often referred to as Schiffreagent and have been widely used to detect aldehydes (Lillie 1977). These components have been incorporated into a solid medium which can be used to identify clones expressing enzymes that produce aldehydes. Indicator plates were prepared by adding 8 ml of pararosaniline (2.5 mg/ml of 95% ethanol) and 100 mg of sodium bisulfite to precooled Luria agar. The plates were stored at room temperature, away from fumes containing aldehydes and light, both of which promote increased background colour.

RESULTS

Cloning of luciferase components

a) Background

The *luxAB* gene products from *X. luminescens* are thermally more stable (up to 45°C) than those of other bacteria (only up to 30°C - Szittner and Meighen, 1990). The *lux* genes of this species were therefore used in the construction of this invention. The order of the *lux* structural genes in this organism is *luxCDABE*, with the luciferase genes flanked by the *luxCD* and *luxE* genes (see Fig. 1).

b) Cloning of *lux* AB genes

In the absence of a commercially available promoter detecting *lux* system, work was undertaken to develop such a system. The promoterless *luxAB* gene cassette is designed to contain unique cloning sites (*Eco*RV, *Cla*I and *Eco*RI) situated immediately upstream from the ribosomal binding site (RBS) of the *luxA* gene, flanking transcriptional terminators (rrnbT_1T_2), and at minimum two unique enzyme sites (*Bam*HI and *Xho*I or *Sal* I or *Pst* I) which would facilitate its cloning into other vectors. Promoter sequences inserted at the *Eco*RV, *Cla*I and *Eco*RI sites and in the correct orientation, have the potential to express the *luxAB* genes.

The purpose of the rrnbT_1T_2 terminators upstream of the *Eco*RV site is to prohibit transcription from other promoters into continuing into the *lux* genes, while those distal to the *lux* genes assist in preventing the destabilisation of the host-vector system.

To facilitate the cloning of the rrnbT_1T_2 terminators in the different positions on pSP72, two sets of PCR primers were designed. Initially the rear terminator was amplified from the vector pKK232-8 (Brosius, 1984) and cloned into the *Bam*HI/*Xba*I site of pSP72, resulting in pT2. *Bgl*II and *Bam*HI produce compatible cohesive ends, however the religated ends cannot be recleaved by either enzyme. Hence, the original *Bam*HI and *Bgl*II restriction sites in pSP72 and the rrnbT_1T_2 PCR product respectively do not exist in pT2. The forward transcriptional terminator was similarly amplified and cloned into the *Bgl*II/*Eco*RV site of pT2. A positive clone (designated pTER) was isolated, characterised and retained for further study.

The *luxAB* genes from *X. luminescens* were amplified and cloned into the *Eco*RI/*Sma*I site pUC18, resulting in a transcriptional fusion between the vector's *lacZ* promoter and the *luxAB* genes. Bioluminescence of colonies

carrying this construct were easily visualised in the dark with the naked eye, on addition of exogenous aldehyde. Characterisation of constructs isolated from ten randomly picked colonies were found to be identical to each other and genuine. One clone, designated pluxAB, was retained (see Fig. 2).

c) Cloning the Aldehyde synthase genes

The *luxCDE* genes were placed under the transcriptional control of the *trc* promoter which contains the *trp* (-35) region and the *lac* UV5(-10) region separated by 17 base pairs (Amann *et al.*, 1988). The PCR fragment generated by the *luxCD* primers was restricted with *Eco* RI/*Kpn* I and ligated to the *Kpn* I/*Bam* HI restricted *luxE* PCR product. Unique restriction enzyme sites were incorporated and positioned at the 5' termini of each primer. The nucleotide sequence of the primers and the conditions of amplification are listed in the appendix. The resulting ligation mixture was subjected to an additional 25 rounds of amplification with the *luxCD* forward and *luxE* reverse primers. The final product comprising *luxCDE* (approx. 3.7 Kb) was cloned into the *Eco*RI/*Bam*HI sites of the expression vector pTrc 99A and transformed into NM522. Positive aldehyde producing clones were isolated by patching transformants onto aldehyde indicator plates. Subsequent PCR and RE analysis of randomly picked clones, verified the authenticity of the constructs. A clone designated pLuxCDE (see Fig. 3), was retained for additional work.

The plasmid pTrc 99A contains the strong *trc* promoter which is inducible by IPTG. Furthermore, the vector contains the *lac*Z ribosomal binding site and the lacI^q repressor, which controls the promoter. The *trc* promoter, however, is a very strong promoter and has been shown to initiate transcription at a low level even in an uninduced state (Amann *et al.*, 1988).

d) Control Construct - Luciferase operon

Constructs encoding the entire lux operon as a single unit, were to serve as a control. The entire lux operon (CDABE genes) was amplified from *X. luminescens* with the *luxCD* forward and *luxE* reverse primers, using Boerhinger 's expand^{TM} Long Template PCR kit. Following *Eco*RI and *Bam*HI restriction, the PCR fragment was cloned into the *Eco*RI/*Bam*HI site of pTrc 99A, resulting in a transcriptional fusion between the *trc* promoter and the *lux* operon. Visual examination of the resulting recombinant clones failed to yield bioluminescence in the absence or presence of exogenous aldehyde. However, luminescence was detected with the aid of the

scintillation counter in the absence of exogenous aldehyde. Characterisation of these transformants via RE and PCR analysis, confirmed that the entire lux operon had been cloned. A representative of the transformants, designated pluxCDABE, was retained for additional work.

To rule out the possibility of an internal promoter activating transcription of the lux operon, the PCR fragment was cloned into the EcoRI/SmaI sites of pTER. Examination with the naked eye and the scintillation counter, of the resulting recombinant clones (designated pTlux), failed to yield bioluminescence in the absence or presence of exogenous aldehyde. pTlux contains the *luxCDABE* genes flanked two transcriptional terminators (outlined in the Fig. 4).

Construction of BTEX sensor

a) Background

The Tol plasmid of *Pseudomonas putida* encodes for enzymes required in the complete degradation of toluene, *m*-xylene and *p*-xylene (Wang & Dunn, 1974; Worsey & Williams, 1975). The genes encoding the degradation of toluene are organised in two operons (see Fig. 5). The 'upper' pathway is responsible for the oxidation of toluene and xylenes to aromatic carboxylic acids (Franklin *et al*, 1983). In addition to the genes for catabolic enzymes, the Tol plasmid encodes for the *XylR* regulatory protein which in concert with upper pathway effectors (such as toluene) triggers expression from the pathway operon promoter (Pu, Abril *et al.*, 1989).

b) Cloning the regulatory genes

The *xylR* gene was amplified (via PCR) from the archetypal Tol plasmid, and individually cloned into the *PstI/Bam*HI site of the broad host range vector pRK404 (Ditta *et al*, 1985). The resulting constructs are under the transcriptional control of the inducible *lacZ* promoter, as shown in Fig. 6.

Since the *xylR* gene contains an internal *PstI* site, an *NsiI* restriction site was introduced at the 5' end of the *xylR* gene product via the forward PCR primer. This primer contains a recognition site for *NsiI*. Cleavage with *NsiI* produces compatible cohesive ends with *PstI*, thus facilitating ligation. The resulting construct, designated pxylac-R, contains the open reading frame (ORF) of the *xylR* gene.

The entire *xylR* gene (including its promoter - P_T) was also cloned. The rationale for creating this construct was to demonstrate that strains containing heterologous genes would respond to BTX compounds more

rapidly than strains harbouring archetype genes. The entire *xylR* gene (2.1 kb) was amplified (via PCR) from the Tol plasmid, and cloned into the *PstI/Bam*HI site of pRK404. To rule out transcriptional interference from the lacZ promoter, the *xylR* gene was cloned in the opposite orientation. This was assisted by exchanging the restriction sites of the forward and reverse PCR primers. Thus, the forward primer contains a *Bam*HI recognition site at the 5' end and similarly the reverse primer contains an *Nsi*I site. A diagram illustrating the features of the resulting construct pXylR, is depicted in Fig. 7.

c) Fusing the catabolic promoters to the lux detection system

The next step of this objective was to clone the promoter of the upper pathway of the Tol plasmid into pTER. The promoter was amplified from the Tol plasmid, individually ligated into the *Eco*RV/*Eco*RI sites of pTER and transformed into *E.coli* HB101. Following PCR and restriction endonuclease analysis, two clones were isolated and retained for future work. The construct harbouring the Pu promoter is designated pTU (refer to Fig. 8).

Having established that the *luxAB* genes from *X. luminescens* were functionally transcribed in *E.coli*, the genes were subsequently cloned into pTU. The *luxAB* PCR fragment was restricted with *Eco* RI following blunting, and individually ligated into the *Eco* RV/ *Eco* RI site of pTU, generating pTLU. Visual examination of the resulting recombinant clones failed to yield bioluminescence in the presence of exogenous aldehyde. Luminescence, however, was detected with the aid of the scintillation counter. A loop full of cells were scraped of the agar plate and resuspended in an eppendorf tube containing 100 ml of Luria broth plus 1% tetradecyl aldehyde. The culture was transferred to polystyrene micro-titre plates and scanned for light production using the scintillation counter. Characterisation of these transformants via RE and PCR analysis, verified that the constructs were authentic.

A diagram illustrating the respective position of the construct's components is shown in Fig. 9.

d) Transcription of Pu promoter constructs

To confirm the potential usefulness of pTLU, luciferase activity of these clones was examined in response to the known effector m-xylene. *E.coli* strains containing the clone and its specific regulator (pxylacR) were also examined (see Table 4). In *E.coli* NM522 containing pTLU a relatively low level of luminescence was detected. These results are in agreement with

previous findings (Hugouvieux-Cotte-Pattat*et al.*, 1990), which have shown that Pu can initiate transcription in the absence of its regulator.

Expectedly, *XylR*-dependent expression of the Pu promoter increased from 40 to 470 fold, depending on the type of sugar and/or IPTG present. A 5 to 10 fold decrease in light output was observed in the absence of the effector molecule (m-xylene), for all organisms except for those grown with glucose. The highest xylene-induced luciferase activity was recorded with cells grown in the presence of glucose (7.5×10^5 LCPS). Some caution must be exercised in interpreting these results, since glucose is known to repress transcription from the lacZ promoter (*XylR* is transcribed from the lacZ promoter).

Table 4: Luminescence activities of recombinant *E.coli* (NM522) strains containing the TOL *xylR* regulators and the 'upper' 'promoter luxAB construct.

Constructs	Supplements	LCPM
pLuxAB	+ IPTG	6.2×10^6
pLuxAB	- IPTG	8.9×10^5
pxylacR	+IPTG, + xylene	-[a]
pxylacR	+IPTG, - xylene	-[a]
pTLU	+IPTG, + xylene	1.6×10^3
pTLU	+IPTG, - xylene	2.3×10^3
pxylacR-pTLU	+ xylene	6.6×10^4
pxylacR-pTLU	- xylene	1.8×10^4
pxylacR-pTLU	+IPTG, + xylene	4.3×10^5
pxylacR-pTLU	+IPTG, - xylene	8.6×10^4
pxylacR-pTLU	+ glucose, + xylene	7.5×10^5
pxylacR-pTLU	+ glucose, - xylene	4.2×10^5
pxylacR-pTLU	+ lactose, + xylene	3.4×10^5
pxylacR-pTLU	+ lactose, - xylene	4.8×10^4

Overnight cultures of the recombinant strains were diluted 100-fold into LB broth containing ampicillin and tetracycline at a concentration of 100 and 10 mg/ml, respectively. The carbon source, glucose and lactose was provided at a concentration of 1 %. To induce expression of the regulatory gene XylR, IPTG was added at a concentration of 1 mM. Luminescence activities (Luminescence Counts Per Second) were determined after 5 hr in the presence of the effector m-xylene (1 mM) and the addition of 2 ml of 10% tetradecylaldehyde. Cultures were grown at 30°C.

-[a], Not detected

e) BTEX sensor control constructs

The lux operon (control construct) was placed under the control of the Pu promoter. As previously mentioned, the entire lux operon (CDABE genes) was amplified from *X. luminescens* using Boerhinger 's expand^{TM} Long Template PCR kit. The PCR fragment, restricted with EcoRI at the 5' end and blunt at the 3' end, was ligated into the EcoRI/SmaI sites of pTu. Following transformation into NM522, positive bioluminating clones were identified in the absence of exogenous aldehyde with the scintillation counter. Restriction endonulease and PCR analysis was used to determine their authenticity. A representative of the transformants, designated pLu-x, was retained for additional work (see Fig. 10). An attempt was made to determine the background luminescence activity. pLu-x was cultured in Luria broth and assayed according to the protocol described in the method and materials section. No luminescence was detected in the presence or absence of exogenous m-xylene.

f) Construction of complete BTEX sensor

The Pu-luxAB cassette was isolated from pTLU via agarose gel electrophoresis following restriction with *BamHI/PstI*, and cloned into the similarly restricted sites of pluxCDE. Characterisation of these transformants via RE and PCR analysis, confirmed that the Pu-luxAB cassette was fused to the *luxCDE* genes. A representative of the transformants, designated pLEU, was retained for additional work. The relative position of the Pu-luxAB cassette in pLEU is outlined in Fig. 11. In order to determine the background luminescence activity, pLEU was cultured in Luria broth and assayed according to the protocol described in the materials and methods section. Repeated assays failed to show luminescence, even in the presence of m-xylene. These results clearly indicate lack of induction of Pu in the absence of the regulatory protein Xyl R.

The test strain Leu-lacR was created by transforming both pLEU and pxylac-R into NM522 and selecting for resistance to ampicillin and tetracycline. Similarly, Leu-R, Lux-lacR and Lux-R were produced by transforming the respective constructs (refer to Tables 1 and 2) into NM522. Assay results of these strains are presented in Fig. 12. and Fig. 13.

The functionality of the novel sensor of the present invention is demonstrated in Figs. 12 and 13. The response of the new sensor was clearly superior to that obtained with the reference construct based on the native lux

gene cassette. The induction of the light signal was faster and much more intensive in the new sensor. Fig. 13 clearly shows that linear calibration curves can be obtained with such sensors down to sub-ppm levels in the case of xylene. Sensor responses are therefore significantly improved by splitting of lux genes as achieved in the novel sensor constructs.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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Appendix 1:

PCR of the *XylR* gene from the TOL plasmid in *Psuedomonas putida*.

According to computer analysis (PCRprimer and Amplify) the following primers were used:

***XylR* gene**

***XylR* ORF:**

Forward primer (R_F): 30 mer

5' 3'

CCAATGCATTGTGGATCATCCCGATAAAAA

Nsi I

Tm - 52.0°C G.C. - 40%

Reverse primer (R_R): 28 mer

5' 3'

CGGGATCCCGCCCGTTTTCACACAACCT

Bam HI

Tm - 60.9 °C G.C. - 60%

PCR conditions: primers annealed for 30 secs. at 46 °C, extention time of 2.5 mins. for 30 cycles.

Resulting product size: Approx. 1963 bps

***XylR* entire gene:**

Forward primer (XylRN.for): 29 mer

5' 3'

CGGGATCCCGGATCTGCGTTGAGGTGGAT

BamHI

Tm - 62.4^{0}C G.C. - 62%

Reverse primer (XylRn.rev): 29 mer

5' 3'

CCAAATGCATTGCCCGTTTTCACACAACCT

NsiI

Tm - 60.9 ^{0}C G.C. - 48%

PCR conditions: primers annealed for 30 secs. at 45^{0}C, extention time of 2.5 mins. for 30 cycles.

Resulting product size: Approx. 2111 bps

Terminators

PCR the *Escherichia coli* 5s ribosomal RNA terminators (rrnbT_1T_2) from the vector pkk232-8. According to computer analysis (PCRprimer and Amplify) the following primers were designed:

Forward Terminator

Forward primer (Frnb.F): 36 mer

5' 3'

GAAGATCTTCGGGATCCTCAGAAGTGAAACGCCGTA

Bgl II Bam HI

Tm - 59.1 ^{0}C G.C - 50%

Reverse primer (Frnb.R): 27 mer

5' 3'

CGGATATCCTGATGCAAAAACGAGGCT

Eco RV

Tm - 62 ^{0}C G.C - 50%

Product size: Approx. 390 bps

Rear Terminator

Forward primer (Rrnb.F): 26 mer

5' 3'

GAAGATCTCAGAAGTGAAACGCCGTA

Bgl II

Tm - 59.1 ^{0}C G.C - 50%

Reverse primer (Rrnb.R): 29 mer

5' 3'

GCTCTAGAGCCTGATGCAAAAACGAGGTC

Xba I

Tm - 62 ^{0}C G.C - 50%

Product size: Approx. 380bps

PCR conditions for both products: primers annealed for 10 secs. at 63 ^{0}C, extention time of 30 secs. for 20 cycles.

Xenorhabdus luminescens Lux CDABE genes

PCR the *lux* operon (*CDABE*) genes (including the ribosomal binding site) from *X. luminescens*.. The following gene(s) were amplified as a single unit:

- *lux CD*
- *lux AB*
- *lux E*
- entire operon *lux CDABE*

According to computer analysis (PCRprimer and Amplify) the following primers were used:

Lux CD:

Forward primer (luxCD.F): 26 mer

5' 3'

GGAATTCCCCCCGATTAAATGGATGGC

Eco RI

Tm - 62.2 ^{0}C G.C. - 53%

Reverse primer (luxCD.R): 32mer

5' 3'

GGGGTACCCCGCAAAAAGTTTCCAAATTTCAT

KpnI

Tm - 57.4 ^{0}C G.C. - 27%

PCR conditions: primers annealed for 30 secs. at 52 ^{0}C, extention time of 2.5 mins. for 25 cycles.

Resulting product size: Approx. 2483 bps

Lux AB:

Forward primer (lux.F): 32 mer

5' 3'

CGGAATTCCGAAGGACTCTCTATGAAATTTGG

Eco RI

Tm - 54.4 ^{0}C G.C. - 36%

Reverse primer (lux.R): 18 mer

5' 3'

CCTCGCTGCAACTCGAAAT

Tm - 61.5 ^{0}C G.C. - 53%

PCR conditions: primers annealed for 30 secs. at 50 ^{0}C, extention time of 2.0 mins. for 25 cycles.

Resulting product size: Approx. 2114 bps

Lux E:

Forward primer (luxE.F): 32 mer

5' 3'

GGGGTACCCCCTTGAGGAGTAAAACAGGTATG

KpnI

Tm - 53.4 ^{0}C G.C. - 41%

Reverse primer (luxE.R): 30 mer

5' 3'

GCAAGGGATCCACTTACAATTAGGCAAAGG

Bam HI

Tm - 58.3 ^{0}C G.C. - 41%

PCR conditions: primers annealed for 30 secs. at 45 ^{0}C, extention time of 2.0 mins. for 25 cycles.

Resulting product size: Approx. 1205bps

Lux operon:

Forward primer (luxCD.F): 25 mer

5' 3'

GTTGGAATTCCCCCCGATTAAATGG

Eco RI

Tm - 62.2 ^{0}C G.C. - 53%

Reverse primer (luxE.R): 30 mer

5' 3'

GCAAGGGATCCACTTACAATTAGGCAAAGG

Bam HI

Tm - 58.3 ^{0}C G.C. - 41%

PCR conditions: Used the Boerhinger expand^{TM} Long Template PCR kit. Essentually the first 10 cycles were as follows: - denature 94^{o}C for 10 secs.
- anneal at 52^{o}C for 30 secs.
- elongation at 68^{o}C for 5 mins.

Then 15 cycles as follows: - denature 94^{o}C for 10 secs.
- anneal at 52^{o}C for 30 secs.
- elongation at 68^{o}C for 5 mins. with a 20 sec incremental increase in each elongation step.

Resulting product size: Approx. 5831 bps

Upper toluate catabolic pathway promoter region

PCR the upper toluate catabolic pathway promoter (including the ribosomal binding site) from the TOL plasmid in *Pseudomonas putida*. According to computer analysis (PCRprimer and Amplify) the following primers are to be used:

Forward primer (U_F): 19 mer

5' 3'

GGAAAGCGCGATGAACCTT

Tm - 62.6 ^{0}C G.C. - 53%

Reverse primer (U_R): 28 mer

5' 3'

GCGAATTCCTGAAGGGTCACCACTATTT

Eco RI

Tm - 61.5 ^{0}C G.C. - 53%

PCR conditions: primers annealed for 10 secs. at 58 ^{0}C, extention time of 30 secs. for 25 cycles.

Resulting product size: Approx. 246bps

Appendix 2:

Examples of promoters which may be used as sensor elements:

Promoter	Signal	Reference
mer	heavy metals (particularly mercury)	Selinofova, O.; Burlage, R. and Barkay, T., Appl. Env. Microbiol., 59, 3083-3090, 1993.
Psal	naphthalene, salicylate	Heitzer, A.; Webb, O. F.; Thonnard, J. E. & Sayler, G. S..Appl. Env. Microbiol., 58, 1839-1846, 1992.
fliC	aluminium	Guzzo, J.;Guzzo, A. & DuBow, M. S. Toxicol. Lett. 64/65, 687-693, 1992.
arsB	arsenic	Corbisier, P.; Ji, G.; Nuyts, G.; Mergeay, M. & Silver, S. FEMS Microbiol. Lett., 110, 231-238, 1993.
cadA	cadmium	Corbisier, P.; Ji, G.; Nuyts, G.; Mergeay, M. & Silver, S. FEMS Microbiol. Lett., 110, 231-238, 1993.
Pm and *Ps*	aromatic hydrocarbons	Abril, M. A., Michan, C., Timmis, K. N., and Ramos, J. L. *J. Bacteriol. 171*, 6782-6790 , 1989.
tcb	chlorobenzenes	van der Meer, J. R., deVos, W. M., Harayama, S. & Zehnder, A. Microbiol. Rev., 56, 677-694, 1992.
clc	chlorobenzoates	van der Meer, J. R., deVos, W. M., Harayama, S. & Zehnder, A. Microbiol. Rev., 56, 677-694, 1992.

bph deVos, W.	polychlorinated biphenyls	van der Meer, J. R., M., Harayama, S. & Zehnder, A. Microbiol. Rev., 56, 677-694, 1992.
cat	catechol	van der Meer, J. R., deVos, W. M., Harayama, S. & Zehnder, A. Microbiol. Rev., 56, 677-694, 1992.
dmpR	phenols	Sze, C. C.; Moore, T. & Shingler, V. Appl. Env. Microbiol., 178, 3727-3735, 1996.
lac	lactose	Engebrecht J.; Simon, M. & Silverman, M. Science, 227, 1345-1347, 1985.
ara	arabinose	Engebrecht J.; Simon, M. & Silverman, M. Science, 227, 1345-1347, 1985.
rha	rhamnose	Ramos, J. L.; Rojo, F. & Timmis, K. N. Nucleic Acids Res. 18, 2149-2152, 1990.
mel	melibiose	Ramos, J. L.; Rojo, F. & Timmis, K. N. Nucleic Acids Res. 18, 2149-2152, 1990.
xyl	xylose	Ramos, J. L.; Rojo, F. & Timmis, K. N. Nucleic Acids Res. 18, 2149-2152, 1990.
sor	sorbose	Ramos, J. L.; Rojo, F. & Timmis, K. N. Nucleic Acids Res. 18, 2149-2152, 1990.
hut	histidine	Fisher, S. H.; Rohrer, K. & Ferson, A. E. J. Bact., 178, 3779-3784, 1996.
nifA	nitrogen assimilation	Shingler, V. Mol. Miocrobiol. 19, 409-416, 1996.
ntrC	nitrogen-assimilation	Shingler, V. Mol. Miocrobiol. 19, 409-416, 1996.

laf	expression of lateral flagella in *E. coli*, induced in response to surfaces	Engebrecht J.; Simon, M. & Silverman, M. Science, 227, 1345-1347, 1985
proU	osmolarity	Zhang, X.; Fletcher, S. A. & Csonka, L. N. J. Bact. 178, 3377-3379, 1996.
csiA	starvation	Kjelleberg, S.; Albertson, N. Flärdh, K.; Holmquist, L.; Jouper-Jaan, Å; Marouga, R.; Östling, J.; Svenblad, B. & Weichart, D. Antonie van Leeuwenhoek, 63, 3/4, 333-342, 1993.
sigB, ctc, gsiB, gspA, katE, gspB, gtaB, csbA	stress response of *Bacillus subtilis*	Hecker, M.; Schumann, W. & Völker, U. Mol. Microbiol., 19, 417-428, 1996.
dnaK	heat shock	Van Dyk, T. K.; Majarian, W. R.; Konstantinov, K. B.; Young, R. M.; Dhurjati, P. S. & LaRossa, R. A. Appl. Env. Microbiol., 60, 1414-1420, 1994.
grpE	heat shock	Van Dyk, T. K.; Majarian, W. R.; Konstantinov, K. B.; Young, R. M.; Dhurjati, P. S. & LaRossa, R. A. Appl. Env. Microbiol., 60, 1414-1420, 1994.
algB	alginate biosynthesis	Shingler, V. Mol. Miocrobiol. 19, 409-416, 1996.
cspA	cold shock	Nakashima, K.; Kanamaru, K.; Mizuno, T. & Horikoshi, K. J. Bact. 178, 2994-2997, 1996.
hrpS	virulence determinants	Shingler, V. Mol. Miocrobiol. 19, 409-416, 1996.

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CLAIMS:

1. A genetic construct for use in a biosensor comprising:

(a) a first nucleic acid molecule including a sequence encoding a reporter molecule having a detectable activity; and

(b) a second nucleic acid molecule including a sequence encoding an enzyme which produces a substrate for the reporter molecule, the first sequence being under the control of a first inducible promoter and the second sequence being under the control of a second inducible promoter.

2. The construct according to claim 1 such that the first nucleic acid molecule encodes bacterial luciferase Lux AB or a functional equivalent thereof, and the second nucleic acid molecule encodes Lux CDE enzyme fatty acid reductase or a functional equivalent thereof.

3. The construct according to claim 2 such that the nucleic acid molecules encoding Lux are obtained from any of the *lux* operons of bioluminescent microorganisms which belong to the genera *Vibrio*, *Xenorhabdus*, *Photorhabdus* and *Photobacterium*.

4. The construct according to any one of claims 1 to 3 for use to detect xylene wherein *Pu* promoter is the first promoter.

5. The construct according to claim 4 substantially as set out in Figure 14.

6. The construct according to any one of claims 1 to 5 such that the first promoter is inducible by exposure to an environmental signal, wherein induction being achieved directly by the signal or indirectly by activating one or more separate pathways in a cell containing the construct.

7. The construct according to any one of claims 1 to 6 further including nucleic acid molecules encoding auxiliary element or elements required for activation of the reporter molecule via its promoter.

8. A biosensor for measuring an environmental signal comprising a cell including the genetic construct according to any one of claims 1 to 7 and a means for measuring the activity of the reporter molecule in the cell when the cell has been exposed to the environmental signal.

9. The biosensor according to claim 8 wherein the cell is selected from the group consisting of bacterial, yeast, fungal and other plant cells, and animal.

10. The biosensor according to claim 9 wherein the cell is a bacterial cell.

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11. The biosensor according to claim 10 wherein the bacterial cell is *Escherichia coli*.

12. The biosensor according to any one of claims 8 to 11 wherein the environmental signal is selected from the group consisting of pollutants, toxins, temperature, irradiation, biological, and chemical.

13. The biosensor according to any one of claims 8 to 12 wherein the means for measuring the activity of the reporter molecule in the cell is an instrument capable of measuring light output of the cells.

14. The biosensor according to claim 13 wherein the instrument capable of measuring the light output of the cells is selected from the group consisting of photomultipliers, charge coupled devices, luminometers, photometers, fiber-optic cables, and liquid scintillation counters.

15. A method of detecting an environmental signal comprising:

(a) exposing a biosensor according to any one of claims 8 to 14 to the signal such that the signal induces the expression of the reporter molecule of the cells in the biosensor, wherein prior to or during exposure the cells are induced to produce an enzyme such that substrate is formed in the cells;

(b) allowing the reporter molecule to react with the pre-formed substrate to form a detectable reaction; and

(c) detecting the reaction.

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