Photochemistry and Photobiology, 2010, 86: 1071–1075

Design and Characterization of an Aequorin-based Bacterial Biosensor for Detection of Toluene and Related Compounds

Mehdi Zeinoddini, Khosro Khajeh*, Farida Behzadian, Saman Hosseinkhani, Ali-Reza Saeedinia and Hesam Barjesteh

Department of Biochemistry, Faculty of Biological Science, Tarbiat Modares University, Tehran, Iran

Received 8 April 2010, accepted 19 May 2010, DOI: 10.1111/j.1751-1097.2010.00775.x

ABSTRACT

An aequorin-based *Escherichia coli* strain JM109 biosensor was constructed and characterized for its potential to detect toluene and related compounds in aqueous solutions. The biosensor was constructed based on a *PGL2* plasmid carrying the lower pathway promoter (Pu) of the xyl operon of *Pseudomonas putida mt-2*, which was incorporated with transcriptional activator xylR and fused to aequorin cDNA named *pGL2*-aequorin. Binding of xylR protein to a subset of toluene-like compounds activates transcription at the Pu promoter, thus expression of aequorin is controlled by xylR and Pu. In this work we have compared the effect of Shine-Dalgarno (SD) and T2 rrnβ terminator sequence in the expression of aequorin. According to the sensitivity of aequorin and increase in the signal-to-noise ratio, this reporter enzyme has reasonable sensitivity compared with other reporter systems. The results indicate higher expression of aequorin in the presence of SD and T2 rrnβ. The activity of aequorin in recombinant whole-cell biosensor was linear from 1 to 500 μM of toluene. The bioluminescence response was specific for toluene-like molecules, so this biosensor cells would be able to detect toluene derivative contamination in environmental samples, accurately.

INTRODUCTION

The petroleum products and water-soluble aromatic components (*e.g.* benzene, toluene, ethylbenzene and xylene) cause serious environmental contamination that is a problem in many countries. Contamination of drinking water can pose a potential health hazard that leads to central nervous depression and skin cancer (1–3). The environmental pollution risks caused by aromatic compounds were determined using conventional analytical methods such as HPLC or gas chromatography. However, these chemical methods are not able to distinguish between available (potentially hazardous) and nonavailable (potentially nonhazardous) fractions of organic compounds to biological systems (4). On the other hand, many different species of soil and water-borne bacteria have evolved networks of enzymes by which complex organic compounds are broken down into metabolic intermediates (5–7). So, these enzymes could be used as bioreporters to construct a whole-cell biosensor. Bioreporters contain two essential genetic elements; a promoter and a reporter gene (8–12).

The degradation of toluene by bacteria has been studied by several laboratories, resulting in the identification of four pathways in *Pseudomonas*. The best-characterized of these pathways is encoded by the TOL plasmid of *Pseudomonas putida mt-2* (13). This plasmid is very large, about 117 kbp in size, approximately 40 kbp of which is needed for the catabolic pathway and the regulatory genes. The catabolic genes of the TOL plasmid are organized into two operons, conveniently referred to as the upper and lower pathways. The upper pathway (xylCAB) encodes the modification of toluene and xylenes to benzoate and toluates. The lower pathway (xylDLEGFJKIH) encodes the degradation of benzoate and toluates to acetaldehyde and pyruvate (14–17). In the TOL operon the binding of toluene and related compounds to the transcriptional activator (xylR) results in an activating interaction between xylR and the promoter sequence (termed Pu). So, this operon can be used to design biosensor cells by fusing the reporter gene to the upstream sequence of the Pu promoter (18,19). In the TOL operon, a second transcriptional activator, xylS, binds benzoate and activates transcription of enzymes responsible for breakdown of benzoate to Krebs cycle intermediates (16,20,21). So, the presence of xylS as well as xylR in this bacterial whole-cell biosensor enables us to detect metabolites such as benzoate.

In previous studies, the most frequently used reporter genes in whole-cell biosensor included *lac*Z, *gfp*, *luc*FF, *lux*AB and *lux*CDBE (4,9,17,18). But in the current research, we have used aequorin, a photoprotein which was first isolated from luminescent jellyfish *Aequorea victoria*. Because it is biologically harmless and sensitive to Ca^{2+}, aequorin is widely used as an intracellular Ca^{2+} indicator (22,23). This photoprotein consists of an apoprotein (apoenzyme) with 189 amino acids, a chromophoric unit (coelenterazine) that acts as a substrate and molecular oxygen (24). When a trace of Ca^{2+} (10^{-7} M) is added to aequorin, conformational change leads to the conversion of coelenterazine to coelentramide and CO_2 with the concomitant emission of an instant flash of blue light (λ_{max} = 469–470 nm) in less than 10 s (25,26). Aequorin could be incorporated into human B cell lines for the detection of pathogenic bacteria and viruses known as the CANARY assay (Cellular Analysis and Notification of Antigen Risks and Yields) (27). In contrast to other bioluminescence molecules, the formation of aequorin is a slow process, whereas the

*Corresponding author email: khajeh@modares.ac.ir (Khosro Khajeh)

luminescence reaction is very fast. Thus the aequorin response occurs as a flash type that decays exponentially and its sensitivity is better than other fluorescent and phosphorescent molecules (28).

Here, we report a photoprotein aequorin-based *Escherichia coli* strain JM109 biosensor that in exposure to toluene contamination emits a blue flash type photon according to the expression of aequorin. The results indicated that the activity of the enzyme in aequorin-based *E. coli* strain JM109 biosensor was linear from 1 to 500 μM of toluene. Therefore, it can be an alternative to conventional chemical methods.

MATERIALS AND METHODS

Chemicals and media. Materials were obtained from the following sources: *E. coli* DH5α, *pET21a*(+) vector, from Novagen; *E. coli* JM109, from Promega; *Tag* and *Pfu* DNA polymerase, *Eco*RI, *Nde*I restriction endonucleases, *T4* DNA Ligase, from Roche; Agarose, from Gibco; LB, from Scharlau; Coelenterazine *hcp*, from Sigma; Tris, $CaCl_2$, dithiothreitol (DTT), disodium ethylenediamine tetraacetic acid (EDTA), sodium phosphate, glucose, lactose, sodium dodecyl sulfate (SDS), toluene, from Merck; all chemicals were of the highest grade available.

Synthetic aequorin. The synthetic gene sequence was designed according to the wild type aequorin mRNA sequence (accession number: AY601106). This gene was cloned into the *Eco*RV site of *pBluescriptII SK*(−) by Bio S&T company (Toronto, Canada).

Construction of the biosensor plasmid. The base vector for engineering of the aequorin-based biosensor cells was *pGL2-px* that was engineered into *pGL2* (Promega) previously (data not shown). The *pGL2-px* is a bioluminescence reporter plasmid for toluene-based compounds including the *xylR* gene, with its promoter (Pr), fused at the *pfl*MI site. On the other hand, an *E. coli rrnB* (T2) terminator sequence (29) was inserted at the *Kpn*I site in the Pu promoter. *xho*I, Shine–Dalgarno (SD) (30) and *pfi*MI were also inserted at the 5′- and 3′-end of aequorin cDNA, respectively. PCR technique was carried out using *pfu* DNA polymerase, a forward primer, 5′-ACT CGA GTA AGG AGG TTG GTA AAA TGC TGT ACG ATG TAC CAG ATT ATG C-3′ containing *xho*I and SD sites and a reverse primer, 5′-TGC CAT AGG TTG GTT ATG GTA CCG CGC CGC CGT AC-3′ containing a *pfi*MI site. In the next phase, the *pGL2-px* and PCR products were digested with *xho*I and *pfi*MI, respectively. Ligation was then carried out by *T4* DNA ligase. The resulting vector was named *pGL2*-aequorin, and transformed into *E. coli* strain DH5α or JM109 cell.

Determination of toluene concentration. Different concentrations of toluene in ethanol were added to media containing the bacterial sensor. As the treatment of *E. coli* with toluene leads to a dramatic decrease in cell viability (31), less than 20 μL of toluene was added to 5 mL of cell culture. In order to determine the precision of toluene concentration, it was also determined by reverse-phase HPLC with a Knauer instrument. Reverse-phase HPLC of toluene was carried out with a vertex B952161 column (250 × 4 mm) filled with Eurospher-100 C18 (particle diameter 10 μm). Acetonitryl (50%) at a flow rate of 1.0 mL min^{-1} was used as the mobile phase. Toluene was detected by monitoring A_{254} by a UV detector.

Induction of aequorin by toluene. *E. coli* cells with *pGL2*-aequorin vector were grown at 37°C in 5 mL of Luria-Bertani (LB) medium containing 100 μM ampicillin. The cells were allowed to grow in the log phase. After reaching an OD_{600} of 0.5–1, the cells were induced by different concentrations of toluene (or related compounds). The induction was repeated every hour for 2 or 4 h. The induced cells were then collected by centrifugation (10 000 ***g*** for 5 min) and resuspended in buffer 1 (50 mM Tris, 10 mM DTT, 5 mM EDTA, pH 8). In the next phase, 5 μM of coelenterazine *hcp* was added to 25 μL whole cell. After an hour's incubation at 4°C, the relative luminescence unit was determined before and after the addition of 100 μL buffer 2 (50 mM Tris, 15 mM $CaCl_2$, 5 mM EDTA, pH 8) using a Sirius-single tube luminometer (Berthold Detection Systems, GmbH).

RESULTS AND DISCUSSION

Constructional characterization of the aequorin-based bacterial biosensor

A map of the plasmid *pGL2*-aequorin is shown in Fig. 1. In this research four plasmid were prepared as follows: (1) *pGL2*-aequorin without SD and *rrnB* sequence ($SD^-/rrnB^-$); (2) *pGL2*-aequorin without SD and with *rrnB* sequence ($SD^-/rrnB^+$); (3) *pGL2*-aequorin with SD and *rrnB* sequence (named $SD^+/rrnB^+$); and (4) *pGL2*-aequorin with SD and without *rrnB* sequence (named $SD^+/rrnB^-$). For this purpose, the PCR product of aequorin was digested with *Xho*I and *Pfi*MI restriction enzymes and ligated to *pGL2-px*.

Determination of the time-dependent induction of aequorin activity

The time course of aequorin activity in DH5α cells harboring $SD^+/rrn\beta^+$ plasmid is shown in Fig. 2. Cells were exposed to 5 μM toluene and assayed for aequorin activity at the time indicated in Materials and methods. The result indicates that the optimum time required for induction of aequorin response to toluene was 150 min.

Determination of aequorin activity in whole-cell biosensor

At first, the activity of aequorin was determined in the presence of different concentrations of coelentrazine *hcp* using the *E. coli* strain JM109 harboring the $SD^+/rrn\beta^+$ plasmid, which was induced by 100 μM toluene. The results revealed that 5 μM was an optimum concentration of coelenterazine *hcp* as a substrate (data not shown).

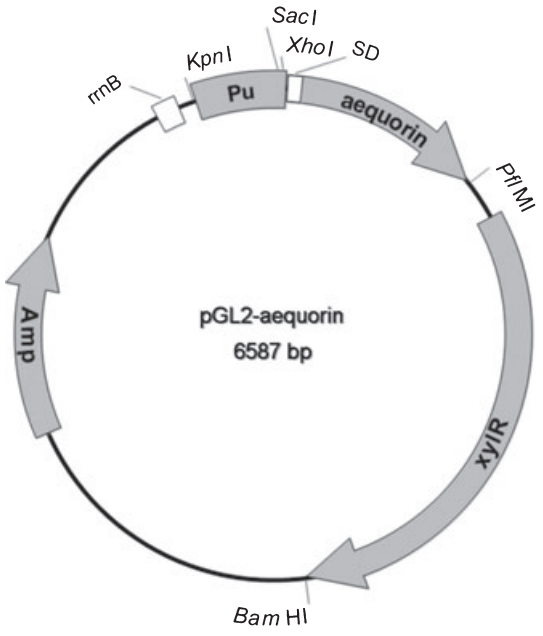

Figure 1. Schematic presentation of the *pGL2*-aequorin plasmid, used for expression of apoaequorin. Important features of the toluene biosensor are indicated, including the location of the Pu promoter, the *Escherichia coli rrnB* transcriptional terminator sequence, the aequorin gene, Shine–Dalgarno (SD) sequence and ampicillin-resistant gene (Amp).

The toluene bacterial biosensors of two *E. coli* strains (DH5α and JM109) were compared with each other. The data showed that the *E. coli* JM109 (aequorin)-based biosensor was much better than the *E. coli* DH5α (aequorin)-based biosensor (Fig. 3). JM109 cells could be considered as an ideal host for many molecular biology applications and are available for convenient transformation in two efficiencies: high efficiency at greater than 10^8 cfu μg^{-1} and sub-cloning efficiency at greater than 10^7 cfu μg^{-1} (32).

In addition, toluene-induced aequorin expression in *E. coli* strain JM109 harboring four prepared plasmids ($SD^-/rrn\beta^-$, $SD^-/rrn\beta^+$, $SD^+/rrn\beta^-$, $SD^+/rrn\beta^+$) was examined. The response of these biosensor cells to toluene is shown in Fig. 4. An SD sequence (30), as ribosome binding site, was inserted in the upstream region of the gene in order to increase the expression of aequorin. A transcription terminator T2 rrnβ sequence (29) was inserted in the upstream region of the Pu promoter to decrease the background luminescence (see map of the pGL2-aequorin in Fig. 1). According to Fig. 4, the presence of both SD and T2 rrnβ leads to higher bioluminescence response. Insertion of T2 rrnβ sequence in the upstream region of the Pu promoter increased the expression which reflects the process known as promoter occlusion (30,33).

The bioluminescence sensor strain *E. coli* JM109 (aequorin)-based biosensor appeared to be more sensitive to toluene, and displayed a response to lower (< 1 μM) concentrations of toluene (Fig. 5). Bioluminescent proteins, such as aequorin, produce light photons through a biochemical reaction and do not require optical excitation. Therefore, problems associated with autofluorescence are eliminated and the ratio of signal to noise promotes (34,35). We have also used coelenterazine *hcp* that was reported to have the best luminescence, with both a high quantum yield and a fast response time (36).

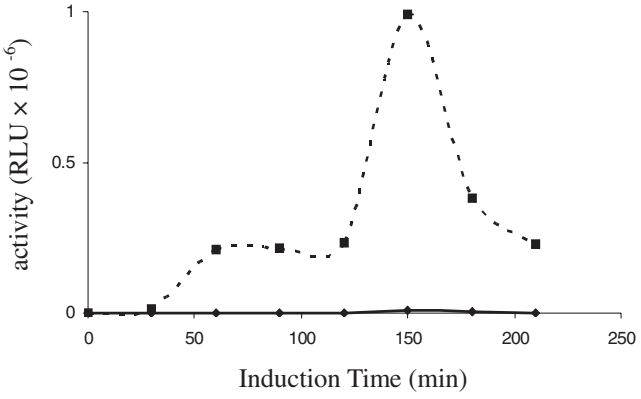

Figure 2. The time course of bioluminescence activity of DH5α cell transformed with *pGL2*-aequorin. Cells were induced by 5 μM toluene and then aequorin activity was assayed before (solid line) and after (dashed line) the addition of calcium solution (for more details see Materials and methods).

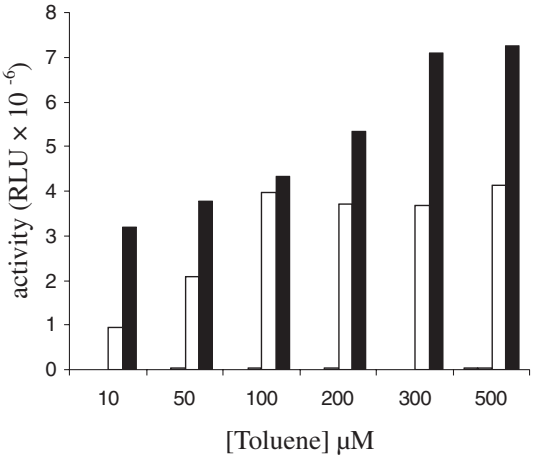

Figure 4. Comparison of maximum bioluminescence activities of whole cell for four plasmids. *E. coli* JM109 cells harboring $SD^-/rrn\beta^-$ (■), $SD^-/rrn\beta^+$ (■), $SD^+/rrn\beta^-$ (□) and $SD^+/rrn\beta^+$ (■) induced in the presence of different concentration of toluene. (Activity of *E. coli* JM109 cells harboring $SD^-/rrn\beta^-$ and $SD^-/rrn\beta^+$ was very low and therefore is not shown in this graph.) The bioluminescence activity of aequorin was recorded after addition of calcium solution (see Materials and Methods).

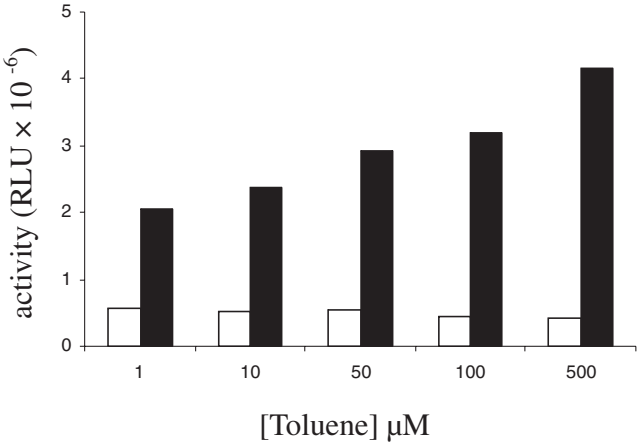

Figure 3. Comparison of maximum bioluminescence activities of cell suspension between two strains of *Escherichia coli* cells harboring the $SD^+/rrn\beta^+$ plasmid. DH5α (□) or JM109 (■) cells were induced in the presence of different concentrations of toluene. The bioluminescence activity of aequorin was recorded after the addition of calcium solution (see Materials and methods).

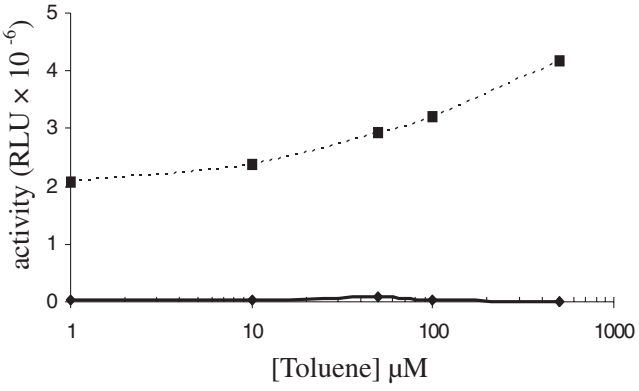

Figure 5. Dose-dependent induction of *Escherichia coli* JM109 (aequorin)-based biosensor by toluene. *Escherichia coli* JM109 cells harboring the $SD^+/rrn\beta^+$ plasmid were determined after 3 h incubation with various concentrations of toluene. The bioluminescence activity of aequorin was recorded before (solid line) and after (dashed line) the addition of calcium solution.

Table 1. Comparison of standard and unknown samples of toluene using bacterial biosensor and HPLC (see Materials and methods).

Samples	Bacterial biosensor	HPLC
Standard (10 μM)	9.8	10.3
Standard (400 μM)	394.0	412.0
Unknown (μM)	0.6	0.6
Unknown (μM)	293.5	302.4

In previous studies, the commonly used bacterial strain was *E. coli* DH5α; reporter enzymes were either luciferase or green fluorescence protein and the toluene detection was between 7.5 and 25 μM (4,15,18). Here, aequorin was used as a reporter enzyme and the detection limit obtained for toluene was less than 1 μM (Fig. 5). Therefore, this aequorin-based biosensor is more sensitive in comparison with other bacterial biosensors.

Finally, toluene concentration in two unknown samples was determined using bacterial biosensors and HPLC (Table 1). There was no significant difference between results obtained by the two methods. Therefore, the biosensor assay could be an alternative to chemical methods such as HPLC.

Comparison of aequorin induction with benzene, xylene and toluene

In order to define the substrate specificity and sensitivity of xylR in JM109 cells harboring the $SD^+/rrn\beta^+$ plasmid, we induced this bacterial biosensor with other aromatic compounds such as benzene and xylene at three different concentrations (50, 100 and 200 μM). The level of reporter sensitivity to other related aromatic components is shown in Fig. 6. The bioluminescent bacterial biosensor response to various aromatic compounds was similar. However, xylene was a better inducer.

CONCLUSION

In this study the design and characterization of an aequorin-based whole-cell biosensor for the measurement of aqueous concentration of toluene pollution is described. We addressed the feasibility of using aequorin as a reporter for pollutant biosensor systems. The basis of our biosensor system was *pGL2-px* that was engineered into *pGL2* (Promega) in a previous study. This plasmid contained a promoter sequence (termed Pu), the transcriptional activator (xylR), an SD sequence (30), as ribosome binding site, and an *E. coli* terminator sequence (*rrnβ*) (29) inserted into *pGL2* (see map in Fig. 1). We have investigated the effect of SD and the T2 *rrnβ* sequence in controlling the expression of aequorin in the *pGL2*-aequorin plasmid. The results indicated that the cell biosensor based on aequorin as reporter could be used for the detection of toluene (up to 1 μM) and related compounds. This bioluminescent bacterial biosensor represents a simple, fast, inexpensive and less laborious alternative to conventional HPLC methods for the detection of aromatic compounds.

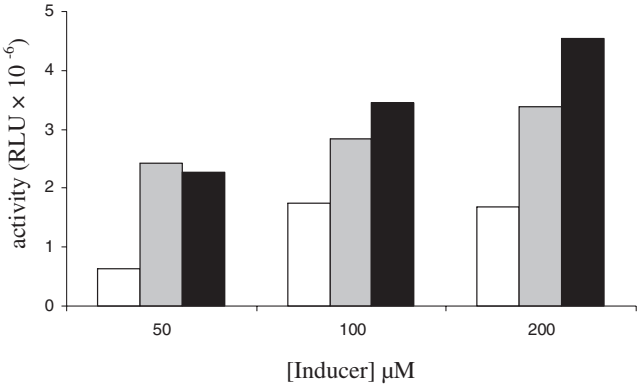

Figure 6. Responses of aequorin-based biosensor to different aromatic compounds. *Escherichia coli* JM109 cells harboring the $SD^+/rrn\beta^+$ plasmid were induced with 50, 100 and 200 μM of toluene (□), benzene (▒) and xylene (■) for 4 h. The bioluminescence activity of aequorin was recorded after the addition of calcium solution (see Materials and methods).

Acknowledgement—We thank the research council of Tarbiat Modares University for financial support.

REFERENCES

1. Dean, B. J. (1985) Recent findings on the genetic toxicology of benzene, toluene, xylenes and phenols. *Mutat. Res.* **145**, 153–181.
2. Dean, B. J. (1978) Genetic toxicology of benzene, toluene, xylenes and phenols. *Mutat. Res.* **47**, 75–97.
3. Chen, H., D. S. Rupa, R. Tomar and D. A. Eastmond (1994) Chromosomal loss and breakage in mouse bone marrow and spleen cells exposed to benzene in vivo. *Cancer Res.* **54**(13), 3533–3539.
4. Li, Y. F., F. Y. Li, C. L. Ho and V. H. C. Liao (2008) Construction and comparison of fluorescence and bioluminescence bacterial biosensors for the detection of bioavailable toluene and related compounds. *Environ. Pollut.* **152**, 123–129.
5. Burlage, R. S., K. Everman and D. Patek (1999) Method for detection of buried explosives using a biosensor. U.S. Patent No. 5, 972,638.
6. Tropel, D. and J. R. Van der Meer (2004) Bacterial transcriptional regulators for degradation pathways of aromatic compounds. *Microbiol. Mol. Biol. Rev.* **68**(3), 474–500.
7. Esteven-Nunez, A., A. Caballero and J. L. Ramos (2001) Biological degradation of 2,4,6-trinitrotoluene. *Microbiol. Mol. Biol. Rev.* **65**(3), 335–352.
8. Daunert, S., G. Barrett, J. S. Feliciano, R. S. Shetty, S. Shrestha and W. Smith-Spencer (2000) Genetically engineered whole-cell sensing systems: Coupling biological recognition with reporter genes. *Chem. Rev.* **7**, 2705–2738.
9. Kohler, S., S. Belkin and R. D. Schmid (2000) Reporter gene bioassays in environmental analysis. *Fresenius J. Anal. Chem.* **6-7**, 769–779.
10. Belkin, S. (2003) Microbial whole-cell sensing systems of environmental pollutants. *Curr. Opin. Microbiol.* **3**, 206–212.
11. Van der Meer, J. R., D. Tropel and M. C. M. Jaspers (2004) Illuminating the detection chain of bacterial bioreporters. *Environ. Microbiol.* **6**(10), 1005–1020.
12. Leveau, J. H. J. and S. E. Lindow (2002) Bioreporters in microbial ecology. *Curr. Opin. Microbiol.* **5**, 259–265.
13. Applegate, B. M., S. R. Kehrmeyer and G. S. Sayler (1998) A chromosomally based *tod-luxCDABE* whole-cell reporter for benzene, toluene, ethylbenzene, and xylene (BTEX) sensing. *Appl. Environ. Microbiol.* **64**, 2730–2735.
14. Olsen, R. H., J. J. Kukor and B. Kaphammer (1994) A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. *J. Bacteriol.* **176**, 3749–3756.
15. Stiner, L. and L. J. Halverson (2002) Development and characterization of a green fluorescent protein-based bacterial biosensor for bioavailable toluene and related compounds. *Appl. Environ. Microbiol.* **68**(4), 1962–1971.

16. Burlage, R. S., S. W. Hooper and G. S. Sayler (1989) The TOL (pWWO) catabolic plasmid. *Appl. Environ. Microbiol.* **55**(6), 1323–1328.
17. Abril, M. A., M. Buck and J. L. Ramos (1991) Activation of the *Pseudomonas* TOL plasmid upper pathway operon. Identification of binding sites for the positive regulator XylR and for integration host factor protein. *J. Biol. Chem.* **266**, 15832–15838.
18. Willardson, B. M., J. F. Wilkins, T. A. Rand, J. M. Schupp, K. K. Hill, P. Keim and P. J. Jackson (1998) Development and testing of a bacterial biosensor for toluene-based environmental contaminants. *Appl. Environ. Microbiol.* **64**(3), 1006–1012.
19. Abril, M. A., C. Michan, K. N. Timmis and J. L. Ramos (1989) Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. *J. Bacteriol.* **171**, 6782–6790.
20. Kong, I. C., H. Suh, Z. Yang and R. S. Burlage (2004) A bioluminescent reporter strain utilizing the lower pathway promoter (Pm) of the *xyl* operon of *pseudomonas*: Optimization of a bioassay for *m*-toluate. *Adv. Environ. Res.* **8**, 647–654.
21. Kaldalu, N., U. Toots, V. D. Lornzo and M. Ustav (2000) Functional domains of the TOL plasmid transcription factor XylS. *J. Bacteriol.* **182**(4), 1118–1126.
22. Shimomura, O., B. Musicki and Y. Kishi (1988) An improved tool for the measurement of calcium ion concentration. *Biochem. J.* **251**, 405–410.
23. Brini, M., P. Pinton, T. Pozzan and R. Rizzuto (1999) Targeted recombinant aequorins: Tools for monitoring $[Ca^{2+}]$ in the various compartments of a living cell. *Microsc. Res. Tech.* **46**, 380–389.
24. Shimomura, O., F. H. Johnson and Y. Saiga (1962) Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. *J. Cell. Comp. Physiol.* **59**, 223–240.
25. Shimomura, O. (1995) Luminescence of aequorin is triggered by the binding of two calcium ions. *Biochem. Biophys. Res. Commun.* **211**, 359–363.
26. Blinks, J. R. (1990) Use of photoproteins as intracellular calcium indicators, environmental health perspectives. *Environ. Health Perspect.* **84**, 75–81.
27. Rider, T. H., M. S. Petrovick, F. E. Nargi, J. D. Harper, E. D. Schwoebel, R. H. Mathews, D. J. Blanchard, L. T. Bortolin, A. M. Young, J. Chen and M. A. Hollis (2003) A B cell-based sensor for rapid identification of pathogens. *Science* **301**(5630), 213–215.
28. Shimomura, O. and F. H. Johnson (1973) Chemical nature of light emitter in bioluminescence of aequorin. *Tetrahedron Lett.* **31**, 2963–2966.
29. Orosz, A., I. Boros and P. Venetianer (1991) Analysis of the complex transcription termination region of the *Escherichia coli rrnB* gene. *Eur. J. Biochem.* **201**, 653–659.
30. Makrides, S. C. (1996) Strategies for achieving high level expression of genes in *Escherichia coli*. *Microbiol. Rev.* **60**(3), 512–538.
31. Jackson, R. W. and J. A. DeMoss (1965) Effect of toluene on *Escherichia coli*. *J. Bacteriol.* **90**(5), 1420–1425.
32. Yanisch-Perron, C., J. Vieira and J. Messing (1985) Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
33. Adhya, S. and M. Gottesman (1982) Promoter occlusion: Transcription through a promoter may inhibit its activity. *Cell* **29**, 939–944.
34. Shimomura, O. and F. Johnson (1969) Properties of the bioluminescent protein aequorin. *Biochemistry* **8**(10), 3991–3997.
35. Inouye, S. (2004) Blue fluorescent protein from the calcium-sensitive photoprotein aequorin is a heat resistant enzyme, catalyzing the oxidation of coelenterazine. *FEBS Lett.* **577**, 105–110.
36. Shimomura, O. (1995) Cause of spectral variation in the luminescence of semisynthetic aequorin. *Biochem. J.* **306**, 537–543.