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Construction of WCB-11: A novel *phi*YFP arsenic-resistant whole-cell biosensor

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Abstract

The prediction and assessment of environmental pollution by arsenic are important preconditions of advocating environmental protection and human health risk assessment. A yellow fluorescent protein-based whole-cell biosensor for the detection of arsenite and arsenate was constructed and tested. An arsenic-resistant promoter and the regulatory gene *arsR* were obtained by PCR from the genome of *Escherichia coli* DH5α, and *phi*YFP was introduced into *E. coli* DH5α as a reporter gene to construct an arsenic-resistant whole-cell biosensor (WCB-11) in which *phi*YFP was expressed well for the first time. Experimental results demonstrated that the biosensor has a good response to arsenic and the expression of *phi*YFP. When strain WCB-11 was exposed to As^{3+} and As^{5+}, the expression of yellow fluorescence was time-dependent and dose-dependent. This engineered construct is expected to become established as an inexpensive and convenient method for the detection of arsenic in the field.

Key words: arsenic; reporter gene; whole-cell biosensor; yellow fluorescent protein (*phi*YFP)
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Introduction

Increasing awareness of the potentially detrimental effects of anthropogenic pollution for human and environmental health has highlighted the need for effective and convenient methods to assess the degree of contamination. Arsenic, an important issue in consideration of public health is a well-known poisonous metalloid that is present in the environment mainly as the result of natural geologic activity (e.g., volcanic activity) and manmade sources (e.g., heavy industry, mining) (Cai and DuBow, 1997). Arsenic is regarded by the WHO as one of the most serious inorganic contaminants in drinking water. Currently, large populations of humans are exposed to arsenic in drinking water (Smith et al., 2000), and long-term exposure is known to induce chromosomal instability and oxidative damage, and to cause neuropathy and liver damage, as well as increase the risk of developing skin, lung, and bladder cancers (Abernathy et al., 1999).

The prediction and assessment of the effects of arsenic on environmental health have become significant preconditions of advocating environmental protection and human health risk assessment. Traditional physical and chemical methods of analysis, such as inductively coupled plasma mass spectrometry (ICP/MS) and gas hydride atomic absorption spectrometry (GHAAS), are precise and simple measurements but they are costly, time-consuming and difficult to implement as on-line monitors. Above all, these methods can not provide information about the bioavailability of contaminants (Verma and Singh, 2005; Lei et al., 2006; Magrisso et al., 2008). There is pressing need for a convenient, real time, low cost method for detection of arsenic and estimation of bio-availability. The establishment of a bioassay-based approach, particularly utilizing microbial whole-cell biosensors to detect and quantify pollutants has become an important subject of current scientific research (Mountfort et al., 2007; Popovtzer et al., 2007).

Because of constant biological evolution, certain microorganisms have evolved genetic determinants that confer a variety of arsenic resistance mechanisms (Silver and Walderhaug, 1992). With the rapid development of molecular biology and genetics, mechanisms of bacterial arsenic resistance have been reviewed several times (Ji and Silver, 1992; Parjit and Rosen, 1992). In microorganisms, the well-characterized arsenic-resistant determinants include the *arsRDABC* operon and the *arsRBC* operon. The *arsRDABC* operon is found in plasmid R46 of *Staphylococcus xylosus*, R773 of *Escherichia coli* and pKW301 of *Acidiphilium multivorum* AIU301 (Tseng et al., 2000; Kann et al., 2005). The *arsRBC* operon which is composed of a regulatory gene (*arsR*) and the structural genes *arsB* and *arsC* (Prithivirajsingh et al., 2001), has been found in

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plasmid pI258 of *Staphylococcus aureus*, plasmid pSX267 of *S. xylosus* and on the chromosome of *E. coli* K-12.

Many attempts have been made to construct whole-cell biosensors that can detect small amounts of arsenic and assess bioavailability by fusing arsenic response elements and regulatory protein genes with various reporter genes (Ron, 2007). Whole-cell biosensors present an alternative to conventional analysis of arsenic and several arsenic biosensors have been described (Roberto et al., 2002; Stocker et al., 2003; Liao and Ou, 2005; Fujimoto et al., 2006). These biosensors mostly use common reporter genes, such as *lac*Z, *luc*, *lux* and *gfp*. Although biosensors based on *lac*Z, *luc* and *lux* are very successful for the detection of arsenic, these methods require additional substrates and/or ATP for signal production. Green fluorescenct protein (GFP) is stable and can complement these defects. Since then, *gfp* has been used extensively to measure biologically relevant concentrations of pollutants. However, the use of *gfp* for whole-cell biosensors has some limitations because fluorophore is formed only slowly after synthesis of the core protein and the high-level background signal limits the application of this reporter. Therefore, there is need to construct and develop more stable, visible and broad host biosensors. Yoshida et al. (2008) developed a pigment gene as a highly sensitive novel reporter to detect environmental pollutants. This biosensor indicates the presence of a pollutant by bacterial colour change, without the need for exogenous substrates or equipment to detect fluorescence or chemiluminescence.

In this study, we used *phi*YFP as a novel reporter system to construct an arsenic-resistant whole-cell biosensor for detection of the bioavailability of As^{3+} and As^{5+}. *phi*YFP is an enhanced variant of the natural fluorescent protein from the jellyfish *Phialidium* sp. (Shagin et al., 2004; www.evrogen.com). It is rather stable to pH changes (p*K*a = 6.0) and is more suitable for the generation of stably transfected cell lines (Wachter and Remington, 1999, http://www.evrogen.com/products/*phi*YFP/*phi*YFP.shtml). However, *phi*YFP codon usage has been optimized for high-level expression in mammals (Haas et al., 1996). In the present study, the arsenic-resistant promoter and the regulatory protein *arsR* were obtained from the genome of *E. coli* DH5α by PCR and, for the first time, *phi*YFP was introduced into prokaryotic *E. coli* DH5α as reporter gene to construct WCB-11, an arsenic-resistant whole-cell biosensor that could express yellow fluorescence after exposure to 8 μmol/L arsenate/arsenite for 2 hr.

1 Materials and methods

1.1 Strains and plasmids

Strains and plasmids are shown in Table 1.

Table 1 Strains and plasmids

Strain/Plasmid	Related genotype	Source of strain
DH5α	*supE*44Δ*lacU*169 (φ80 *lacZ*ΔM15) *hdsR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1*relA*1	Preservation of our study group
BL21 (DE3)	*hsdS gal* (λc*Its*857 *ind*1 S*am*7 *nin*5 *lac* UV-5-T7 gene1)	Tiangen Biotech, China
puc18-luc	Amp^r	Preservation of our study group
phi-Yellow-PRL	Kan^r	Evrogen, Russia
T7-puc18-yfp	Amp^r	This study
ars-puc18-yfp	Amp^r	This study

1.2 Enzymes and reagents

Restriction endonucleases, bacteriophage T4 DNA ligase and Vent DNA polymerase were obtained from New England Biolabs (USA). All chemicals of analytical reagent grade and all media and buffer solutions were prepared using deionized distilled water.

1.3 Construction of biosensor plasmid in *E. coli*

The plasmid vector puc18-luc was used for the construction of the biosensor. We inserted gene of *yfp* instead of *luc* as a reporter gene, and it was amplied by PCR using phi-Yellow-PRL as the template. The PCR primers incorporated *Xho*I and *BamH*I restriction endonuclese cut sites. Additionally, because of the need to express of *yfp*, we inserted an SD sequence (AAGGAG) before the forward primer *yfp*FF: 5'-ACTGCTCGAGAAG GAGATATACATATGAGCAGCGGCGCC-3' and triplet stop codon (CTATCATTA) before the reverse primer *yfp*RF: 5'-TGAGGGATCCCTATCATTATCACAGGTAG GTCTTGCG-3'. The resulting 728 bp fragment was digested with *Xho*I and *BamH*I and ligated with T4 DNA ligase into puc18-luc cut with the same enzymes to form T7-puc18-yfp.

To construct an arsenic-resistant biosensor based on the expression of *yfp*, the entire *arsR* gene and the O/P region were amplified by PCR from the genome of DH5α (Fujimoto et al., 2006) incorporating *Sac*I and *Xho*I restriction endonuclease sites. Then, the DNA amplicon was cloned into T7-puc18-*yfp* to form ars-puc18-*yfp* (Fig. 1). The resulting recombinant plasmid ars-puc18-yfp was transformed into *E. coli* DH5α by the heat shock (42°C) method.

1.4 Cultivation of bacteria and arsenic response assay

A single colony of DH5α (ars-puc18-yfp) was picked from medium containing 100 μg/mL ampicillin and grown at 37°C overnight, then diluted (1:50) in fresh LB with 100 μg/mL ampicillin and incubated at 37°C in an orbital shaker at 225 r/min until the optical density (OD) at 600 nm reached 0.6. Various concentrations of As^{3+} and As^{5+} were added to the bacterial cultures. Each cell culture (200 μL) was transferred to a Multimode Microplate Spectrophotometer (Varioscan Flash with Dispenser, Thermo, USA) for measurement the fluorescence of YFP (λ_{ex} = 517 nm, λ_{em} = 557 nm). The Data are reported as the averages of three replicates.

1.5 Microscopy

The fluorescence of the bacterial cells cultured in LB medium and exposed to arsenic was viewed in a

fluorescence microscope (AxioSkop 40FL, Zeiss, Germany) equipped with a 100 W mercury arc lamp and a set of excitation filters (Bio-Rad, USA).

1.6 Growth curves

A single each colony of *E. coli* DH5α and *E. coli* DH5α (ars-puc18-yfp) were grown overnight in LB medium (DH5α harboring ars-puc18-yfp supplemented with 100 μg/mL ampicillin) until the optical density at 600 nm reached 1.0. After inoculating (1%) the overnight cell culture into fresh LB medium, the cells were grown for an additional 9 hr in the presence of various concentrations of As^{3+} and As^{5+} at 37°C at 250 r/min. Optical density at 600 nm and turbidimetry measurements were performed with a spectrophotometer (Unico UV-2000, USA). At least three independent experiments were conducted for each effector.

2 Results

2.1 Development of arsenic biosensor plasmid

We first searched for a novel reporter gene (*yfp*) similar to *gfp* by PCR amplification. A recombinant plasmid, T7-puc18-yfp, was developed and introduced into *E. coli* BL21 (DE3), as described in Section 1.3. The sensor strains produced yellow fluorescence When cultured in LB medium with 100 μg/mL ampicillin and 1 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG), indicated that the YFP was expressed successfully in bacteria. The O/P region and *arsR* derived from the genome of DH5α was then replaced with the T7 promoter to form ars-puc18-yfp. In the presence of an effecter, DH5α (ars-puc18-yfp) showed a statistically significant change in fluorescence intensity.

2.2 Evaluation of the arsenic-resistant whole-cell biosensor

The sensor strain, DH5α (ars-puc18-yfp), was cultured with 8 μmol/L arsenite for 2 hr before measuring the fluorescence. In our experimental treatment, a positive response of the biosensor strain for arsenite was observed by fluorescence microscopy (Fig. 1).

The induction of yellow fluorescence of the sensor strain was time-dependent when treated with 5 μmol/L arsenite and arsenate (Fig. 2). As shown in Fig. 2, the intensity of DH5α fluorescence (ars-puc18-yfp) increased with induction time, and the background fluorescence was lower at all times. DH5α (ars-puc18-yfp) without arsenite or arsenate supplement was used as the control.

The fluorescence of DH5α cells carrying ars-puc18-yfp giving resistance to arsenic was also dose-dependent. As described above, the response of the DH5α strain toward As^{3+} and As^{5+} was time-dependent. Because an induction period of 2-hr could produce sufficient fluorescence signal for a dose-dependent induction experiment, we used 2-hr of induction for constructing concentration response curves. As shown in Fig. 3, the yellow fluorescence produced by the bacteria increased with increased concentration of As^{3+} and As^{5+}. However, the response is not linear, rather; it is a nearly parabolic distribution over the entire range measured. Moreover, as expected, the induction of yellow fluorescence by arsenite was greater than that induced by arsenate. Interestingly, the fluorescence

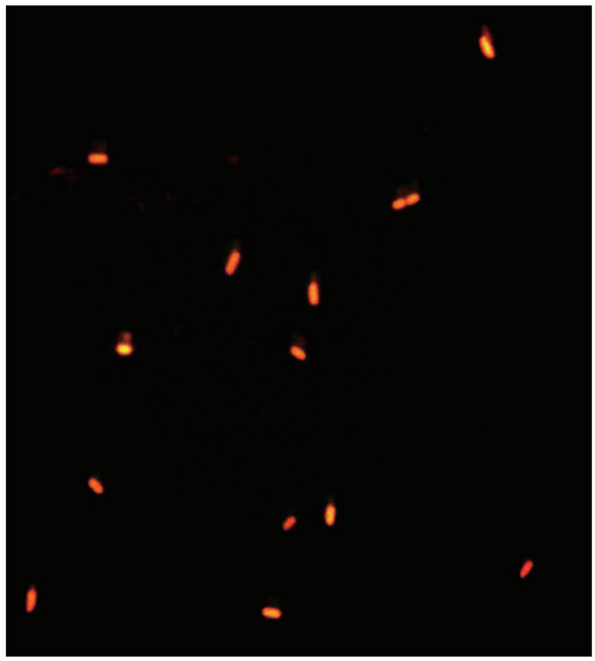

Fig. 1 Fluorescent micrograph of DH5α harboring ars-puc18-yfp and expressing YFP. Image was taken using a 100×/1.3 Oil immersion lens on a Zeiss AxioSkop 40FL fluorescence microscope.

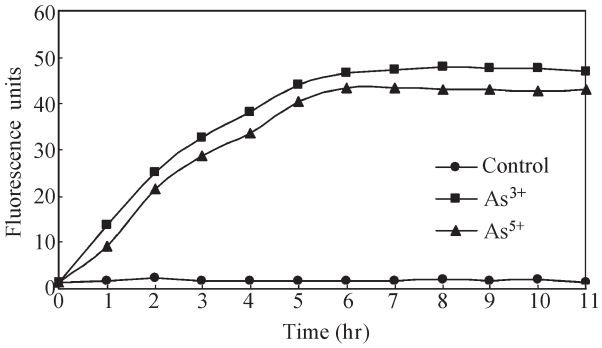

Fig. 2 Induction time course of yellow fluorescence with effectors. The *E. coli* DH5α (ars-puc18-yfp) were cultured to 5 μmol/L As^{3+} and As^{5+} for various intervals at 30°C. Fluorescence measurements were performed in triplicates.

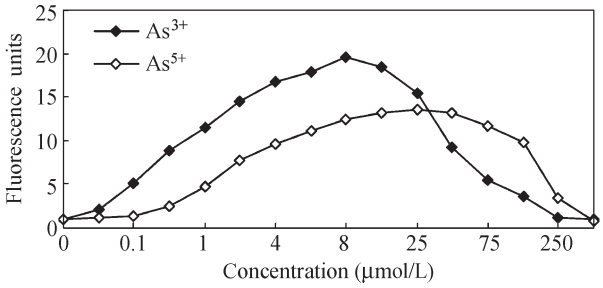

Fig. 3 Assay of the yellow fluorescence produced in response to various concentrations of As^{3+} and As^{5+}. The fluorescence of DH5α (ars-puc18-yfp) was determined after 2-hr induction with various concentrations of As, as described in Section 1.4. The data presented are the mean values of three independent experiments.

intensity with As^{5+} treatment actively increased steadily up to a concentration of 25 μmol/L. In contrast, the yellow fluorescence intensity induced by treatment with As^{3+} increased only up to a concentration of 8 μmol/L and decreased gradually at higher concentrations.

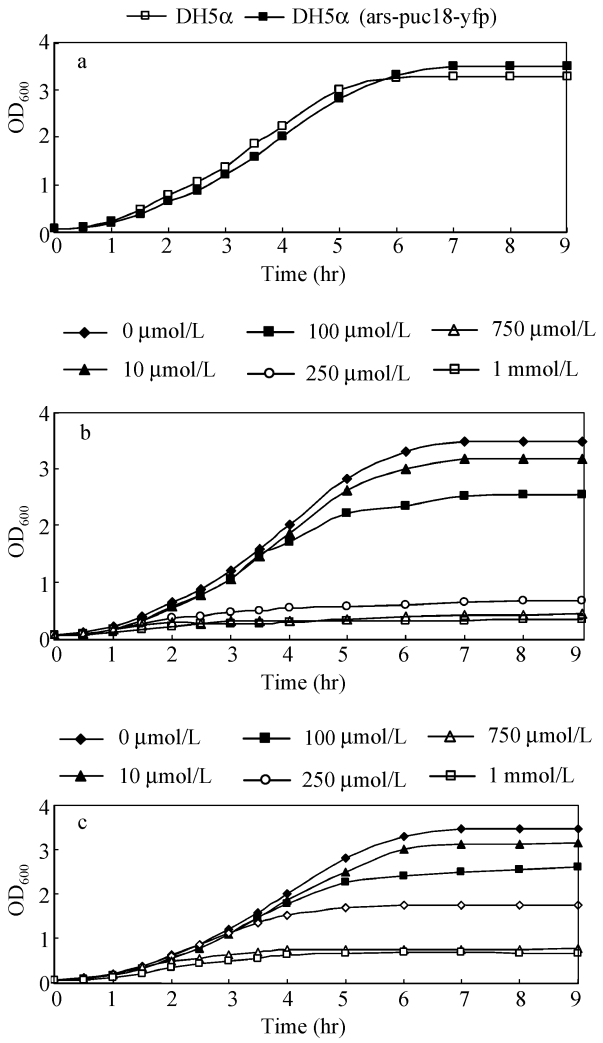

Fig. 4 Assay of the tolerance of the sensor strain with various concentrations As^{3+} and As^{5+}. (a) growth curve of DH5α cells carrying ars-puc18-yfp plasmid; (b) growth curves of DH5α (ars-puc18-yfp) with various concentrations As(III); (c) growth curves of DH5α (ars-puc18-yfp) with various concentrations As(V). The value of the optical density (OD) at 600 nm is the means of three independent experiments.

2.3 Tolerance of biosensor strain

The tolerance range of this biosensor strain was examined to demonstrate the utility of DH5α (ars-puc18-yfp) with arsenic supplements for field-testing. As shown in Fig. 4a, the effect on the growth of DH5α with insertion of the ars-puc18-yfp plasmid was little; only the stationary phase of the sensor strain lagged by 1 hr.

We further examined of various concentrations As^{3+} and As^{5+} on the growth of DH5α cells harbouring ars-puc18-yfp plasmid. The growth of the sensor strain samples with As^{3+} or As^{5+} was similar when the concentration of arsenic was ⩽ 100 μmol/L (Fig. 4b, c); however, the sensor strain showed a significant difference between treatments at concentrations of arsenic ⩾ 250 μmol/L. The difference demonstrated that the tolerance range for As^{3+} was limited compared to that for As^{5+}.

3 Discussion

3.1 Evaluation of the reporter *phi*YFP

Very few attempts have been made to develop novel reporters other than *crtA* from the photosynthetic bacterium *Rhodovulum sulfidophilum* (Fujimoto et al., 2006). In this study, an arsenic-resistant biosensor plasmid was developed for *E. coli* DH5α using *phi*YFP as a novel reporter gene. The sensor strain WCB-11 could clearly respond to arsenic and successfully induce the expression of PhiYFP, which laid a solid foundation for field determination of arsenic.

As the length of the induction time increased, the background expression of the sensor strain stayed at a relatively low level, and the yellow fluorescent protein was stable for a long time (Fig. 2). Therefore, as a natural GFP-like protein, in addition to retaining the inherent merits of GFP (e.g., heat stability), PhiYFP had an obvious advantage in that it could improve on the drawback of GFP, which requires high-level expression (data not shown). In other words, the detection threshold at which WCB-11 produced yellow fluorescence might be lower, thus improving detection sensitivity.

It is well known that reporters can have an effect on the application of a whole-cell biosensor. Table 2 summarizes the features of the commonly used reporters. In the present study, we used *phi*YFP as a novel reporter to construct and test an arsenic-resistance whole-cell biosensor. Our

Table 2 Features of commonly reporter genes for developing whole-cell biosensors

Gene	Protein	Advantages	Disadvantages	Reference
luc	Firefly luciferase	High sensitivity, broad dynamic range, simplicity	Substrate requirement; ATP, O_2 and Mg^{2+} requirement	Lewis et al., 1998; Naylor, 1999
lux	Bacterial luciferase	Rapid response, ease of measurement	Heat labile (> 30°C); O_2 requirement	Naylor, 1999
*lac*Z	β-Galactosidase	Various detection methods, detection by the naked eye	Substrate requirement; complicated colorimetric assays	Daunert et al., 2000; Yagi, 2007
gfp	Green fluorescence protein	Autofluorescence, no substrate requirement, high stability	Delay expression after synthesis of protein; high background signal	Tsien, 1998; Naylor, 1999
crtA	Spheroidenone	Detection only by naked eye, development of new host other than *E. coli*	Low sensitivity; substrate requirement; lag time before determination of induction	Fujimoto et al., 2006; Yoshida et al., 2008

results demonstrated that it is convenient using *phi*YFP as a reporter in determining the bioavailability of arsenic. However, PhiYFP has some negative aspects; for example, it takes some time to produce fluorescence after synthesis of the core protein, which would require long induction or sensitive instrumentation, such as quantitative microscopy or flow cytometry, but, given the currently available technology, the problem is likely to be solved.

3.2 Characteristics of WCB-11 biosensor

As observed in earlier works, the concentration of effectors and the length of induction time were major factors in determining the level of fluorescence intensity. Other factors might influence the expression of whole-cell biosensors, including the identity of the host strains, length of incubation time, composition of the medium, and the growth phase of harvested cells (Tauriainen et al., 1997; Roberto et al., 2002). It has been suggested that ArsC could reduce As^{5+} to As^{3+} in *arsRBC* determinants; thus, the downstream *ars* genes expressed by As^{3+} interact with the *arsR* repressor (Carlin et al., 1995). The plasmid constructed in this study contains *arsR* gene and the O/P region without the *arsC* gene; therefore, the observed response might be derived from the *E. coli* DH5α genome.

As shown in Fig. 4a, the introduction of a recombinant plasmid had a little influence on the growth of the host strain. It is well known that the host strain can be responsible for a series of physiological effects after the recombinant plasmid vector carrying the exogenous DNA fragment has been transformed into it; especially regarding expression of the plasmid vector, the expression of exogenous genes is likely to result in a decreased growth rate of the host cells. However, because of the lower level of expression of PhiYFP, recombinant plasmid ars-puc18-yfp had a little impact on the stability of the host strain. These results suggested that *yfp* can be used as a marker for the development of whole-cell biosensors.

Our results showed that different concentrations of arsenic had considerable influence on the growth of the host strain (Fig. 4b, c); the concentration of arsenic is positive correlated to the toxicity to host cells. These data provide a threshold limit for the measurement of arsenic as a pollutant (the concentration of arsenic must be controlled at a level that cannot significantly inhibit growth of the host strain). Meanwhile, DH5α (ars-puc18-yfp) appeared to have a greater tolerance range for As^{5+} than that of As^{3+}, but there is no reasonable explaination of the difference. Further research is necessary to study the tolerance mechanisms of host cells.

4 Conclusions

In this study, an arsenic-resistant biosensor plasmid was developed for *E. coli* DH5α using the vector T7-puc18-yfp, containing gene *yfp* as a novel reporter, and the DNA region responsible for arsenic resistance in *E. coli*. The introduction of the SD sequence and the triplet stop codon allowed DH5α harbouring ars-puc18-yfp to express the yellow fluorescence protein. This work is the first to use PhiYFP as a reporter in prokaryotic bacteria for detecting arsenic. The results demonstrated that the engineered construct had a good response to arsenic and expression of PHIYFP. When exposed to different levels of arsenic, expression of yellow fluorescence was both time-dependent and dose-dependent; moreover, the non-pathogenic whole-cell biosensor WCB-11 showed different tolerance ranges for arsenite and arsenate. The results presented here suggest a convenient method for the detection of arsenic that could be used in the field.

Like other biosensors, our approach has some problems, including reproducibility, specificity, and accuracy, that need be overcome. However, compared to traditional physical and chemical detection methods, the application of biosensors can provide more accurate data for environmental monitoring. In addition, the relative simplicity and the low cost of whole-cell biosensors serve as sufficient motivation to overcome the defects. It is likely that a whole-cell biosensor will find wide application due to growing concern about the potential dangers of environmental contamination by arsenic.

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