

Development of a broad-spectrum fluorescent heavy metal bacterial biosensor

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Received: 7 March 2012 / Accepted: 2 October 2012 / Published online: 16 October 2012
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Abstract Bacterial biosensors can measure pollution in terms of their actual toxicity to living organisms. A recombinant bacterial biosensor has been constructed that is known to respond to toxic levels of Zn^{2+} , Cd^{2+} and Hg^{2+} . The zinc regulatory gene *zntR* and *zntA* promoter from *znt* operon of *E. coli* have been used to trigger the expression of GFP reporter protein at toxic levels of these ions. The sensor was induced with 3–800 ppm of Zn^{2+} , 0.005–4 ppm of Cd^{2+} and 0.001–0.12 ppm of Hg^{2+} ions. Induction studies were also performed in liquid media to quantify GFP fluorescence using fluorimeter. To determine the optimum culture conditions three different incubation periods (16, 20 and 24 h) were followed. Results showed an increased and consistent fluorescence in cells incubated for 16 h. Maximum induction for Zn^{2+} , Cd^{2+} and Hg^{2+} was observed at 20, 0.005 and 0.002 ppm, respectively. The pPROBE-*zntR*-*zntA* biosensor reported here can be employed as a primary screening technique for aquatic heavy metal pollution.

Keywords Bacterial biosensor · Heavy metals · Zinc · Cadmium · Mercury · GFP

Introduction

Aquatic pollution by heavy metals is posing a major threat to both humans and ecological balance through bioaccumulation,

resulting in poisoning of the food chain [1–4]. Identification and quantification of heavy metals for pollution monitoring normally requires expensive equipment and often needs substantial sample pretreatment. Also such methods cannot distinguish between biologically available and unavailable fractions. Heavy metals in the aquatic environment can only have an impact on living organisms to the extent they are bioavailable. Hence a test of bioavailability is important in bioremediation, waste dumping, waste treatment optimization, environmental impact assessment.

Employing designer microorganisms (Biosensors) for environmental monitoring is a good alternative in this regard. Biosensors refer to the organisms that have been genetically engineered to produce a measurable signal in response to a specific chemical or physical agent in their environment. When whole cell biosensors are employed for this purpose, detection of the target compound is possible in pico molar concentrations and is directly linked to its toxicity [5]. Metal responsive biosensors contain two essential genetic elements, a ‘metal inducible promoter’ and a ‘reporter gene’. In *E. coli*, it has been shown that the *znt* operon codes for proteins that are required for tolerance to Zn^{2+} and Cd^{2+} [6]. The product of *zntR* is a regulatory protein that binds *zntA* promoter in the absence of heavy metals. The product of *zntA* is an ATPase involved in the active efflux of Zn^{2+} , Cd^{2+} , Pb^{2+} , Co^{2+} and Ni^{2+} from *E. coli* [7]. In the presence of metal ions the conformation of ZntR changes allowing the RNA polymerase to transcribe *zntA* [8, 9]. The *zntA* gene can be replaced by a reporter gene to design a recombinant heavy metal biosensor. Ivask et al. [10] used this strategy to develop a luminescent biosensor. However, use of luciferase gene as reporter makes the end-point measurement complex as it requires the addition of substrate for luminescence. With the advent of new

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technologies like fluorescent in situ hybridization or scanning confocal laser microscopy for which bioluminescence is not compatible, the green fluorescent protein (GFP) is increasingly being used to construct whole-cell biosensors [11–17].

In the recombinant biosensor reported here *zntR-zntA* O/P-GFP gene cassette has been introduced on a plasmid vector into *E. coli* DH5 α . Simple fluorescence measurements are well correlated to metal concentrations and can be effectively used for first level screening of aquatic broad spectrum heavy metal pollution.

Materials and methods

Construction of the biosensor plasmid

The *zntA* gene promoter and *zntR* regulatory gene of *E. coli znt* operon were amplified from *E. coli* DH5 α genomic DNA and cloned upstream to the GFP gene in pPROBE-KT vector. Briefly, the *zntA* promoter was amplified from *E. coli* DH5 α genomic DNA using *zntA*-F1 (5'-ATATCCCGGGGAGCCACTATCGCCGACGCTTCC-3') and *zntA*-R1 (5'-TTAAGGTACCCATGGCATCCTCCGGTTAAGTTT-3') primers having linkers for *Sma* I and *Kpn* I sites to facilitate directional cloning. The PCR reaction mix included 40 ng of genomic DNA, 10 pmol each of forward and reverse primers, 200 μ mol each of dNTPs, 0.75 U *Taq* polymerase, 1.5 mM MgCl₂, 1 \times assay buffer in a total volume of 25 μ l. The thermocycler was programmed for 35 cycles of 94 $^{\circ}$ C for 45 s/58 $^{\circ}$ C for 45 s/72 $^{\circ}$ C for 45 s for denaturation, annealing and extension, with initial denaturation at 95 $^{\circ}$ C for 5 min and final extension at 72 $^{\circ}$ C for 8 min. The 193 bp product amplified was purified by extraction from 1.5 % agarose gel (QIAquick Gel extraction kit, Qiagen). The PCR product and pPROBE-GFP vector were digested sequentially with *Sma* I and *Kpn* I restriction endonucleases (NEB, UK), ligated using T4 DNA ligase and transformed into DH5 α competent cells using standard protocols [18]. The recombinants were grown on LB plates containing 50 μ g/ml kanamycin and the plasmid was named pPROBE-*zntA*.

The regulatory element *zntR* was amplified using primers *zntR*-F1 (5'-AATTGCATGCATGTATCGCATTGGTGAGCT-3') and *zntR*-R1 (5'-ATATTCTAGATCAACAACCACTC TTAACGC-3') having linkers for *Sph* I and *Xba* I sites, respectively. PCR reaction mix was prepared as discussed above. The thermocycler was programmed as discussed previously except for annealing temperature, which was kept at 60 $^{\circ}$ C. The 446 bp product amplified was purified by extraction from 1.5 % agarose gel (Gel extraction kit, Qiagen) and cloned into pPROBE-*zntA* plasmid using *Sph* I and *Xba* I restriction endonucleases as

above. The recombinant clone is named pPROBE-*zntR-zntA* (Fig. 1) and the insert sequence was confirmed by sequencing.

Induction of the bacterial biosensor

The expression of GFP was induced by streaking pPROBE-*zntR-zntA* biosensor on LB agar-kanamycin plates containing different concentrations of inducing heavy metal ions, Zn²⁺ (3, 6, 20, 100, 200, 400, 600, 800 mg/l), Cd²⁺ (0.005, 0.01, 0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) and Hg²⁺ (0.001, 0.002, 0.004, 0.008, 0.016, 0.03, 0.06 and 0.12 mg/l). The heavy metal stock solutions of ZnCl₂, CdCl₂ and HgCl₂ were prepared in double distilled water and filter sterilized. Appropriate quantities of these stock solutions were either spread on LB agar (fresh sterile plastic petri plates were used) or added to LB broth to the selective media to obtain the desired inducing ion concentration. Liquid media was prepared in specially cleaned glass test tubes. The test tubes were immersed in 0.1 % HNO₃ at 50 $^{\circ}$ C for 24 h and rinsed with deionized water and dried.

Fluorescence microscopy

The expression of GFP in biosensor bacteria plated on LB-kan agar with different metal ions was observed through fluorescence microscopy. For this, the culture was allowed to grow for 18 h at 37 $^{\circ}$ C and fluorescence was recorded using the Zeiss fluorescence stereozoom microscope at 25 \times . For GFP detection, a bandpass GFP filter set with excitation range 450–490 nm and emission range 500–550 nm was used. The image was recorded directly on a computer using

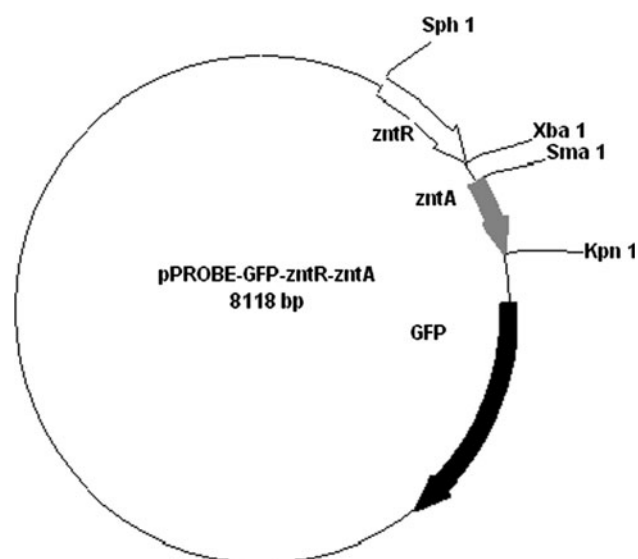


Fig. 1 Schematic representation of the pPROBE-*zntR-zntA* biosensor construct

the Carl Zeiss AxioCam MRC camera and Axiovision 21 Software Version 4.3.

Fluorimetric analysis

The GFP expression at each inducing ion concentration was quantified using fluorimeter. Briefly, the expression of GFP was induced in broth culture of the biosensor by adding Zn^{2+} , Cd^{2+} and Hg^{2+} ions separately at the above mentioned concentrations. To obtain the optimum culture conditions for biosensor assay, the GFP expression was quantified in cultures incubated for 16, 20 and 24 h separately. The cultures were incubated at 37 °C with shaking in an orbital shaker and fluorescence was quantified using the Berthold Fluorimeter, MikroWin 2000. For GFP detection, a GFP filter set with excitation range 480 nm and emission range 530 nm was used. The cell density was measured using a spectrophotometer and normalized across the samples. The GFP fluorescence was quantified directly using Mithras Software 2000.

Results and discussion

In the present study, the Zn^{2+} responsive elements, *zntR* [9] and *zntA* [8] were used as receptor elements controlling the expression of GFP reporter gene for engineering a bacterial biosensor responsive to toxic levels of Zn^{2+} and Cd^{2+} . This receptor system has been used earlier by Ivask et al. [10] to develop a bacterial biosensor, but with luciferase gene as the reporter element. In this study, GFP protein has been used as reporter owing to its high stability, easy scoring and lack of need to add any substrate compared to luminescence-based system [19]. GFP based whole cell biosensors have been developed for arsenic [11], cadmium [13, 14], mercury [15], lead [16] and uranium [17]. However, most of these biosensors developed so far are specific to a single heavy metal. Since, the aquatic environment often is contaminated with more than one heavy metal, it is imperative to have a broad spectrum bacterial biosensor that can detect the presence of more than one heavy metal simultaneously. This will drastically decrease the number of biosensors to be employed for the screening of a water body for the presence of heavy metal pollutants.

Here, we report the development of a recombinant broad spectrum-based bacterial biosensor, pPROBE-*zntR-zntA*. This biosensor construct was tested for inducible expression of GFP on LB-kan media containing 3, 6, 20, 100, 200, 400, 600 or 800 ppm (mg/l) of Zn^{2+} ion, 0.005, 0.01, 0.1, 0.5, 1.0, 2.0, 3.0 or 4.0 ppm of Cd^{2+} ion and 0.001, 0.002, 0.004, 0.008, 0.016, 0.03, 0.06 and 0.12 ppm of Hg^{2+} ion while the media without any inducer ion served as control. GFP fluorescence was seen in cells

induced with Zn^{2+} , Cd^{2+} or Hg^{2+} ions while there was no GFP expression in controls. The induction studies were also performed in liquid media in order to quantify the GFP fluorescence using a fluorimeter. To determine the optimum culture conditions for better performance, the biosensor was subjected to three different incubation periods viz, 16, 20 and 24 h. Results revealed that an incubation period of 16 h gave increased and consistent fluorescence compared to 20 and 24 h. The reduction in fluorescence beyond 16 h of incubation period could be attributed to the half life of GFP protein and cell death due to senescence [20]. In cells exposed to Zn^{2+} , an incubation period of 24 h resulted in a sudden drop in fluorescence beyond 200 ppm for unknown reason. The maximum fluorescence was recorded at 20, 0.005 and 0.002 ppm for Zn^{2+} , Cd^{2+} and Hg^{2+} ions, respectively (Figs. 2, 3, 4). However, Ivask et al. [10] reported the maximum induction at 650 ppm in case of zinc while it is 0.1 and 0.2 ppm in case of cadmium and mercury, respectively. This shows that the use of GFP as reporter protein increased the sensitivity of the bacterial biosensor. To test the effect of inducing metal ions on the growth of sensor bacteria, a growth curve analysis was performed by exposing bacteria to different concentrations of Zn^{2+} , Cd^{2+} and Hg^{2+} ions. Results showed a decline in cell density beyond 200, 0.5 and 0.016 ppm of Zn^{2+} , Cd^{2+} and Hg^{2+} ions, respectively (Fig. 5).

The co-inducibility of the biosensor with Zn^{2+} , Cd^{2+} and Hg^{2+} ions is in agreement with earlier studies of Binet and Poole [21] and Ivask et al. [10] who reported the induction of the *znt* resistance system (*zntR* and promoter of *zntA*) with Zn^{2+} , Cd^{2+} and Hg^{2+} ions. It has been previously observed that ZntA protein of *znt* operon exports, in addition to Zn^{2+} , also other di-valent ions such as Cd^{2+} , Pb^{2+} , Co^{2+} and Ni^{2+} from the cell [7, 22]. However, it is noteworthy that the concentrations of Hg^{2+} , Cd^{2+} and Zn^{2+} needed for the induction of the *zntR-zntA* based sensor differ

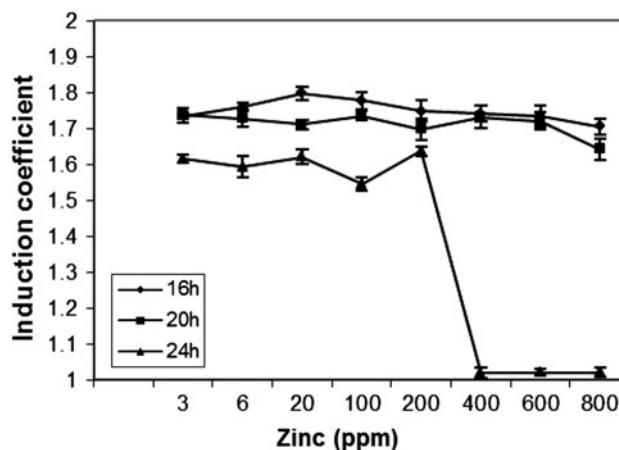


Fig. 2 Fluorescence response of the biosensor exposed to different concentrations of Zn^{2+} ions for 16, 20 or 24 h of incubation period

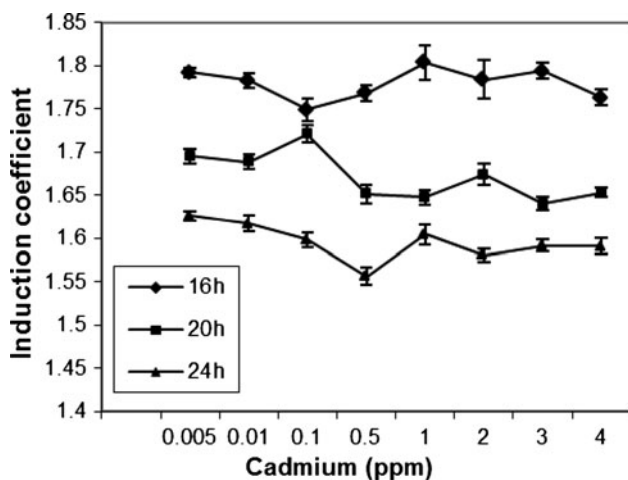


Fig. 3 Fluorescence response of the biosensor exposed to different concentrations of Cd^{2+} ions for 16, 20 or 24 h of incubation period

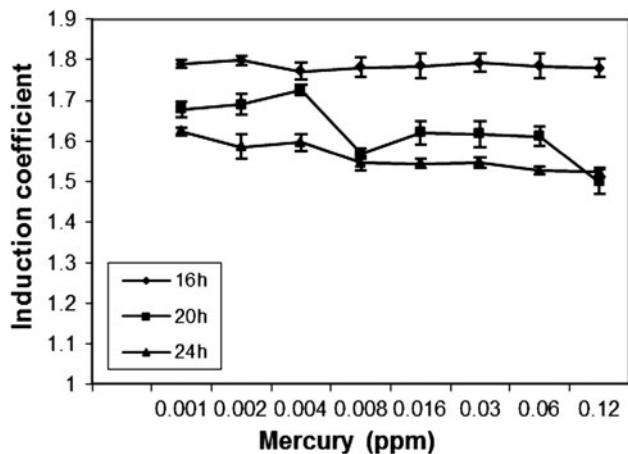


Fig. 4 Fluorescence response of the biosensor exposed to different concentrations of Hg^{2+} ions for 16, 20 or 24 h of incubation period

remarkably, the amount of Hg^{2+} being 700 and the amount of Cd^{2+} being 450 times lower than the respective concentration for Zn^{2+} . Most importantly, the concentrations of mercury, cadmium and zinc inducing the biosensor are in good correlation with the toxicities of these metals. For example, for crustacean *Daphnia magna* the 24 h LC50 values for Hg^{2+} (0.01 mg/l) and for Cd^{2+} (0.64 mg/l; 24) are 760 and 12 times lower than for Zn^{2+} (7.6 mg/l; 25). The correlation of the sensitivities with toxicities of the metals is quite expected as the 'sensing' elements of the current biosensors originate from living organisms.

It can be concluded that the bacterial biosensor, pPROBE-*zntR-zntA* offers a simple method to assess toxic concentrations of Zn^{2+} , Cd^{2+} and Hg^{2+} . This is especially because sub-lethal levels of these heavy metals can be accurately predicted by measuring fluorescence of liquid cultures using a fluorescence reader. This broad spectrum-based biosensor can be employed for primary screening of

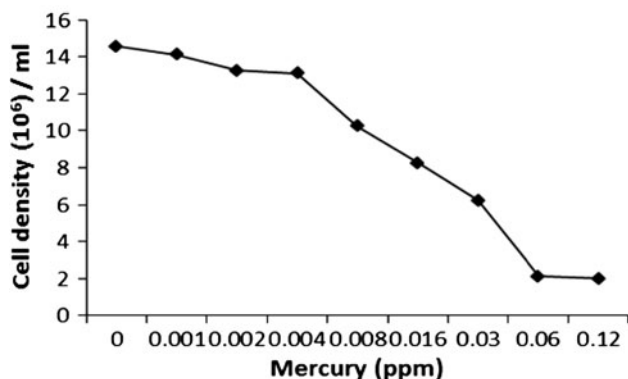
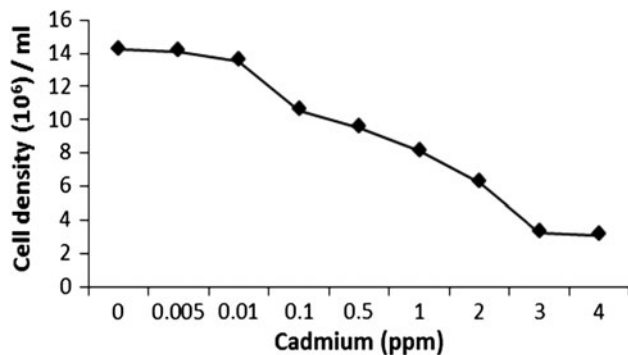
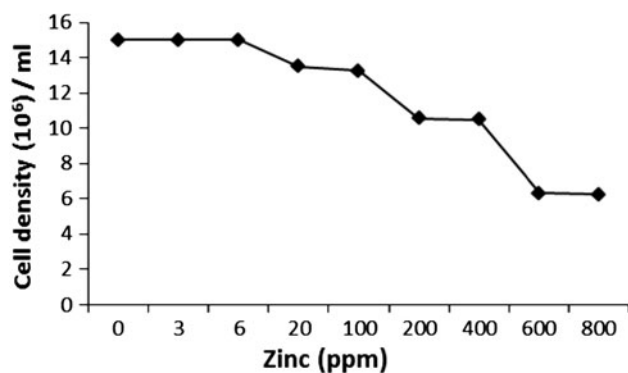


Fig. 5 Effect of different concentrations of Zn^{2+} , Cd^{2+} and Hg^{2+} ions on growth of biosensor bacteria expressed in terms of cell density

naturally polluted water once the methodology is standardized. Therefore, the future challenge lies in using such biosensors to develop portable, inexpensive, single-use tools for monitoring environmental toxicity.

Acknowledgments The authors would like to acknowledge Dr. W. S. Lakra, Director, CIFE and Dr. Dilip Kumar, Former Director, CIFE for providing necessary facilities, ICAR for JRF fellowship to the first author, Dr. Kshitish Majumdar and Dr. S. Ayyappan for their valuable help, advice and support.

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