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A GFP-based bacterial biosensor with chromosomally integrated sensing cassette for quantitative detection of Hg(II) in environment

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Abstract

A mercury biosensor was constructed by integrating biosensor genetic elements into *E. coli* JM109 chromosome in a single copy number, using the *attP/attB* recombination mechanism of λ phage. The genetic elements used include a regulatory protein gene (*merR*) along with operator/promoter (O/P) derived from the mercury resistance operon from pDU1358 plasmid of *Serratia marcescens*. The expression of reporter gene *gfp* is also controlled by *merR*/O/P. Integration of the construct into the chromosome was done to increase the stability and precision of the biosensor. This biosensor could detect Hg(II) ions in the concentration range of 100–1700 nmol/L, and manifest the result as the expression of GFP. The GFP expression was significantly different ($P \leqslant 0.05$) for each concentration of inducing Hg(II) ions in the detection range, which reduces the chances of misinterpretation of results. A model using regression method was also derived for the quantification of the concentration of Hg(II) in water samples.

Key words: *attP/attB* integration; *E. coli* biosensor; GFP; mercury

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Introduction

Heavy metal pollutants produced by various anthropogenic activities finally enter into aquatic ecosystem and pose detrimental effects on aquatic biodiversity, aquaculture production and human and aquatic life. Mercury is one of the most dangerous heavy metals that can potentially harm life even at low concentrations (Langford and Ferner, 1999). Mercury entering into atmosphere as a metallic vapour after oxidation is brought back into freshwater bodies and oceans by rain, where microbes transform it to methyl mercury (Meech et al., 1997) that enters the aquatic food chain and shows bioaccumulation and biomagnification effects (Cabana et al., 1994; Monteiro et al., 1996). Studies on various aquatic organisms of different aquatic habitats consumed by human beings have revealed high mercury concentrations (Colaçco et al., 2006; Houserova et al., 2006; Soundarapandian et al., 2010; Suseno and Wisnubroto, 2009; Taherianfard et al., 2008; Vera et al., 2008). Probably the most infamous case of human toxicity from mercury was the outbreak of Minimata disease in Japan due to consumption of mercury contaminated fish and shellfish (Daher et al., 1999).

Although classical analytical methods can detect most metal ions in environmental sample with great precision (Kamalakkannan et al., 2004), they are elaborate and expensive and also do not differentiate between the unavailable and bio-available fractions. Several metal-specific recombinant bacterial biosensors have been constructed to determine toxic levels of inorganic mercury (Hansen and Søorenson, 2000; Omura et al., 2004; Selifonova et al., 1993; Virta et al., 1995), organomercurial (Ivask et al., 2001), zinc, cadmium, cobalt and mercury (Corbisier et al., 1996). As living beings, their 'measurements' reflect the bio-available concentration rather than the total concentration and can be employed as a 'first detection filter' (Belkin et al., 1997).

All the biosensors reported earlier carry the biosensing gene cassette on plasmids. Since plasmid copy number and stability depend on several factors (Ryan and Parulekar, 1991; Summers and Sherratt, 1984), experimental results employing reporter genes could be misleading (Klotzky and Schwartz, 1987). Obukowicz et al. (1986) found that chromosomal integration of a foreign gene maximizes its stability and minimizes the potential for horizontal transfer of the gene to other bacterial species. The present study was aimed at developing a stable inorganic mercury biosensor that has the biosensor gene cassette integrated

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into the *E. coli* genome in a single copy. The biosensor genetic element was derived from the *mer* operon of pDU1358 plasmid of *Serratia marcescens*, and *gfp* was used as the reporter gene. The λ phage mechanism for *E. coli* genome integration involving *attP* and *attB* recognition sites, which has been developed into an elegant two plasmid vector system by Diederich et al. (1992), was employed for genome integration of the biosensor gene cassette.

1 Materials and methods

1.1 Bacterial strains and plasmids

Plasmid pDU1358 (GenBank Accession No: M24940.1, Nucifora et al., 1989) derived from *Serratia marcescens,* a kind gift of Dr. Anne O. Summers, University of Georgia, USA) was the source of mercury resistance regulatory element (*merR*) and its operator-promoter (O/P). Plasmid pDB402 (kind gift from Dr. Ekker, Mayo Clinic, Minnesota, USA) was used as the source of *gfp* reporter gene. Plasmids pLDR9 and pLDR8 (kind gift from Dr. Lene Juel Rasmussen, Department of Life Science and Chemistry, Roskilde University, Denmark) were used for genome integration. Plamsid pLDR9 carries the *attP* site and ampicillin resistance marker, while pLDR8 is a helper plasmid that expresses λ-integrase and carries kanamycin resistance marker. These plasmids were maintained in *E. coli* JM109 strain. *E. coli* DH5α strain was used for all cloning experiments.

1.2 Construction of biosensor cassette

For integrating the biosensor gene cassette into *E. coli* genome, all the genetic elements *merR*/O/P, *merR*, and *gfp* were first cloned into pLDR9 plasmid that contains the *att*P site (Diederich et al., 1992). A *gfp* fragment of 759 bp was excised from pDB402 plasmid using *Xba* I and *Pst* I. The gel purified fragment (Qiaquick Gel Extraction kit, Qiagen, Germany) was ligated into identical sites of pLDR9 vector and transformed into *E. coli* DH5α cells using standard protocols (Sambrook and Russell, 2001). The recombinant clone was identified using horizontal slot lysis followed by restriction endonuclease digestion and named pLDR9-GFP. The 585 bp *merR*/O/P region was amplified from pDU1358 plasmid using Int-MerR/O/P-F and Int-MerR/O/P-R primers (Table 1) carrying *Eco*RI and *Xba* I sites, respectively. PCR program included an initial denaturation at 95°C for 5 min, 35 cycles of 94°C for 1 min/52°C for 45 sec/72°C for 1 min, and final extension at 72°C for 10 min. The PCR product was purified using PCR purification kit (Qiagen, USA) following manufacturer's instructions and digested with *Eco*RI and *Xba* I. The gel purified fragment was cloned into similarly digested and purified pLDR9-GFP plasmid upstream to the *gfp* gene. The positive clone was selected by colony PCR with *merR*/O/P specific primers and named pLDR9-Hg. Positive clones were further confirmed by restriction digestion and sequencing.

1.3 Integration of biosensor cassette into *E. coli* genome

Chromosomal integration was achieved following the method of Diederich et al. (1992). A *NotI* fragment of pLDR9-Hg plasmid (3167 bp; excluding the origin of replication) was purified from agarose gel, re-circularized and transformed into JM109 cells containing pLDR8 plasmid. Integrase expression was induced in the transformed clones by incubating at 42°C and recombinants were selected on LB-Kan-Amp plates (100 μg/mL and 50 μg/mL of ampicillin and kanamycin, respectively). The non-recombinant clones were eliminated by PCR using attBflank-F and attBflank-R primers (Table 1) that amplify a fragment of 500 bp in the absence of any integration event (Platt et al., 2000). The Int-MerR/O/P-F and attBflank-R primers could amplify a 1.5 kb fragment from the recombinant clones. The positive clone used for further study was named *E. coli* AC-Hg and maintained on LB-Amp plates.

1.4 Induction

Mercuric chloride ($HgCl_2$), zinc chloride ($ZnCl_2$) and cadmium chloride ($CdCl_2$) stock solutions of concentration 50×10^{-3} mol/L (10.6×10^{-12} g/L), 0.5×10^{-3} g/L and 1×10^{-5} g/L, respectively, were prepared in demineralised water and sterilized by autoclaving. Appropriate volumes of stock metal solutions were added to 5 mL LB-amp broth to get the desired inducing concentrations of Hg(II), Zn(II) and Cd(II) ions. *E. coli* AC-Hg mercury biosensor was tested for inducible expression of GFP by exposure to Hg(II) ions in a concentration range from 1 nmol/L to 10 μmol/L. The experiment was done in quadruplicates with uninduced biosensor as negative control. A primary overnight LB-Amp broth culture of *E. coli* AC-Hg (50 μL) was inoculated in the inducing medium and allowed to grow for 12 hr at 37°C with shaking. Subsequently, 200 μL aliquots of each sample were transferred to a microtiter plate for measuring GFP fluorescence in a Multilabel Reader (Mithras LB 940, Berthold Technologies, Germany). A GFP filter set with excitation range 485 nm and emission range 535 nm was used. The GFP fluorescence intensity was measured in terms of RLU (relative light unit, 1 RLU = 43 photons/sec). Absorbance at 600 nm was measured in a biophotometer (Eppendorf AG, Germany) using 60 μL of

Table 1 Primers used in the present study

Sl. No.	Primer name	Sequence(5′–3′)	Length (base)	Annealing temp. (°C)
1	Int-MerR/O/P-F	AAAGAATTCGTCCTCAGCATAGTACCGGGA	30	62
2	Int-MerR/O/P-R	AAATCTAGACATACGCTTGTCCTTTCAAA	29	56
3	attBflank -F	CGCCGCCTGAATCGTGGTATG	21	58
4	attBflank-R	CACTCTCTGGCAAGCGCCTC	20	58

the same aliquots and cell densities were calculated using the equation of $OD_{600} = 2.5 \times 10^8$ cells/mL (Sambrook and Russell, 2001). *E. coli* AC-Hg was also induced with Zn(II) and Cd(II) ions in the range of 3–200 and 0.1–2 ppm, respectively, to check its specificity for Hg(II). Fluorescence and cell density were also measured for *E. coli* JM109 exposed to 0–1000 nmol/L Hg(II) ions to estimate background bacterial fluorescence and effect of Hg(II) ion on its growth. The absolute fluorescence (RLU) at each concentration of the inducing metal ions (Hg(II), Zn(II) and Cd(II)) was calculated by Eq. (1):

$$\mathrm{RLU_{test}} = \frac{X_{\mathrm{tested}}}{\mathrm{OD_{600\text{-}tested}}} - \frac{X_{\mathrm{nc}}}{\mathrm{OD_{600\text{-}nc}}} \quad (1)$$

where, $\mathrm{RLU_{test}}$ means absolute RLU at tested metal(II) concentration, X means observed RLU, tested means at tested metal(II) concentration, nc means negative control.

1.5 Data analysis

Cell density and absolute RLU were estimated for each concentration of Hg(II), Zn(II) and Cd(II) and subjected to one way ANOVA to identify statistically significant variations ($P \leqslant 0.05$). Multiple regression analysis of GFP fluorescence (RLU) and cell density data were done to derive a equation that can be used for estimating the concentration of Hg(II) ions in unknown water samples. The accuracy of the equation was established by testing standard water samples prepared in the lab containing Hg(II) concentrations other than the ones used to derive it.

2 Results and discussion

2.1 Integration of biosensor cassette into *E. coli* genome

The pLDR9-Hg construct carrying *merR*/O/P fragment between *Eco*RI and *Xba*I sites and *gfp* reporter gene between *Xba* I and *Pst* I sites is represented schematically in Fig. 1. Both *merR* and *gfp* genes are bi-directionally expressed from the same operator-promoter. Fig. 2a shows the 3167 bp fragment generated by *Not* I digestion of pLDR9-Hg. This fragment was gel purified, re-circularized and transformed into *E. coli* JM109 containing pLDR8 plasmid. The integration was confirmed by PCR using merR/O/P forward and attBflank reverse primers that amplified 1.5 kb fragment in positive clones (Fig. 2b). A positive recombinant bacterial clone was named *E. coli* AC-Hg and used for induction studies.

2.2 Induction of *E. coli* AC-Hg with mercury

The *E. coli* AC-Hg biosensor responds to Hg(II) ions ranging from 100 nmol/L (21.2×10^{-6} g/L) to 1700 nmol/L (360×10^{-6} g/L). Absolute fluorescence (RLU) at each concentration of inducing Hg(II) ion was estimated using Eq. (1). This value estimates the fluorescence over and above the un-induced biosensor and accounts for any leaky expression of the reporter. The fluorescence values (RLU) of the host bacteria JM109 at different Hg(II) ion concentrations remain unaltered and 10^3 times

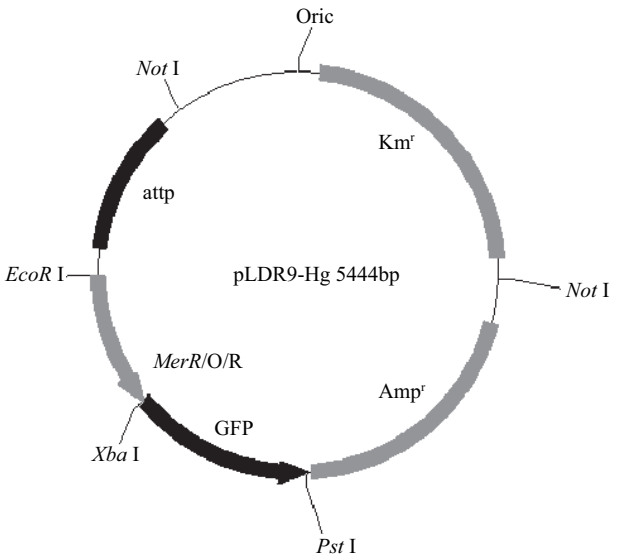

Fig. 1 Schematic representation of pLDR9-Hg construct.

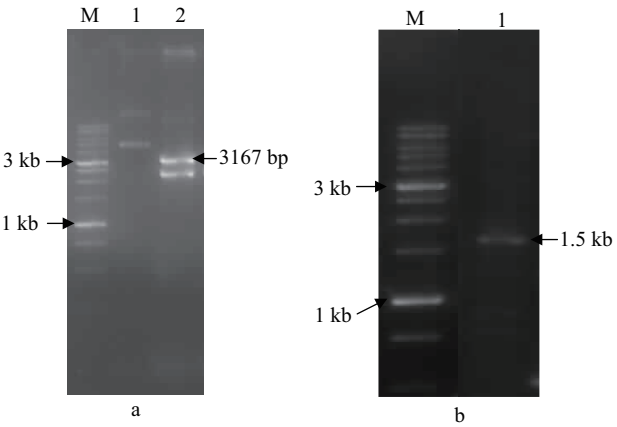

Fig. 2 Construction and confirmation of *E. coli* AC-Hg recombinant. (a) lane M: 1kb DNA ladder; lane 1: pLDR9-Hg uncut plasmid; lane 2: *Not* I digest of pLDR9-Hg plasmid releases fragment of 3167 bp used for integration. (b) Lane M: 1kb DNA ladder; lane 1: a 1.5 kb PCR amplification product of attB-flank reverse and Int-merR/O/P forward primers obtained from genomic DNA of *E. coli* AC-Hg biosensor.

lower than the un-induced biosensor (data not shown). One way ANOVA analysis of Y values showed that GFP fluorescence of the biosensor differs significantly ($P \leqslant 0.05$) at each concentration. The averages of quadruplicate Y values were plotted against the inducing Hg(II) ion concentration (Fig. 3) and the fluorescence emission showed a linear increase from 100 to 1700 nmol/L Hg(II) ions. Beyond 1700 nmol/L Hg(II) the fluorescence emission was non linear (data omitted to get linearity in graph and regression). The linear increase in fluorescence upto 1700 nmol/L is likely to be due to the increased binding of Hg(II) to unoccupied MerR regulatory protein molecules, resulting in the upregulation of GFP expression. Hg(II) ion concentration beyond 1700 nmol/L causes cell death in *E. coli* as recorded by experiments (data not shown). Using other cell types, several researchers have reported that high heavy metal concentrations affect cellular machinery and cause cell mortality (Nakada et al., 1980; Kuznetsov et al., 1986).

Compared to plasmid-based mercury biosensors report-

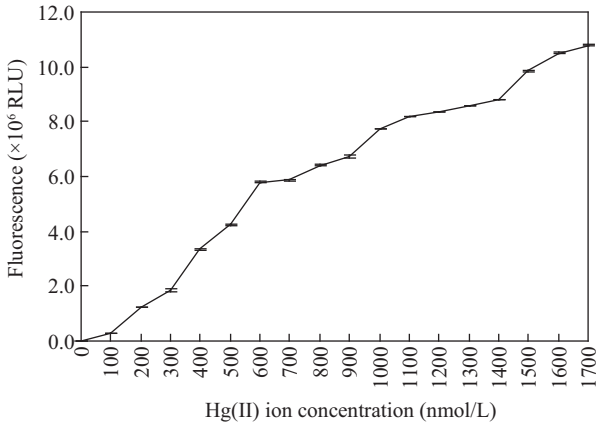

Fig. 3 Average absolute fluorescence of the *E. coli* AC-Hg biosensor at various concentrations of inducing Hg(II) ions.

ed earlier, the upper limit of Hg(II) ion detection is higher for *E. coli* AC-Hg biosensor. Hansen & Søorensen (2000) reported three mercury biosensors with different reporter genes, lux, lac and gfp. While the *mer-gfp* biosensor reported can detect up to 1250 nmol/L, the *mer-lux* and *mer-lac* biosensors detect a maximum of 12.5 and 5 nmol/L Hg(II) ions, respectively. The authors attributed the difference in detection ranges to different induction protocols. The bioluminescent inorganic mercury biosensor developed by Selifonova et al. (1993) detects 1–1000 nmol/L Hg(II) ions. A detection upper limit of 1700 nmol/L Hg(II) with *E. coli* AC-Hg is the highest reported so far.

2.3 Cell density of *E. coli* AC-Hg at different Hg(II) ion concentrations

The *E. coli* AC-Hg biosensor tolerates higher Hg(II) ions concentration compared to mercury sensitive *E. coli* JM109, which shows marked decrease in cell density at 1000 nmol/L Hg(II) ion concentration (Fig. 4). This improvement in mercury tolerance is most likely due to sequestration of Hg(II) ions by the regulatory protein MerR expressed by the biosensor. Mercury resistance mechanisms are not present on the *E. coli* chromosome and are usually associated with the R plasmids found in resistant *E. coli* strains (Ogawa et al.,1984). Mercury sensitive strain (Hg^S) of *E. coli* do not grow beyond 0.3 mmol/L of $HgCl_2$ (Suzuki et al., 1997) and its growth (cell

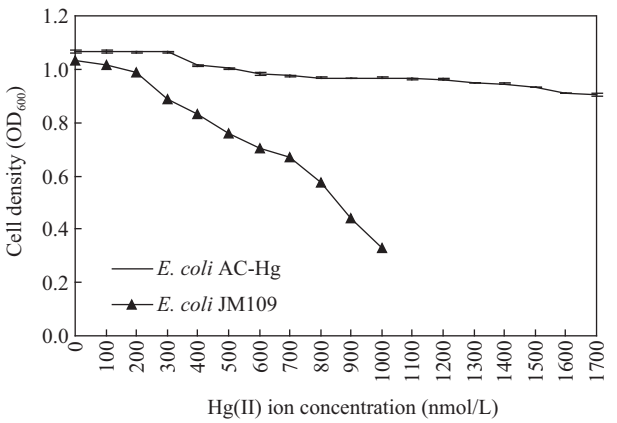

Fig. 4 Average cell density of the *E. coli* AC-Hg biosensor and *E. coli* JM109 at various concentrations of Hg(II) ions.

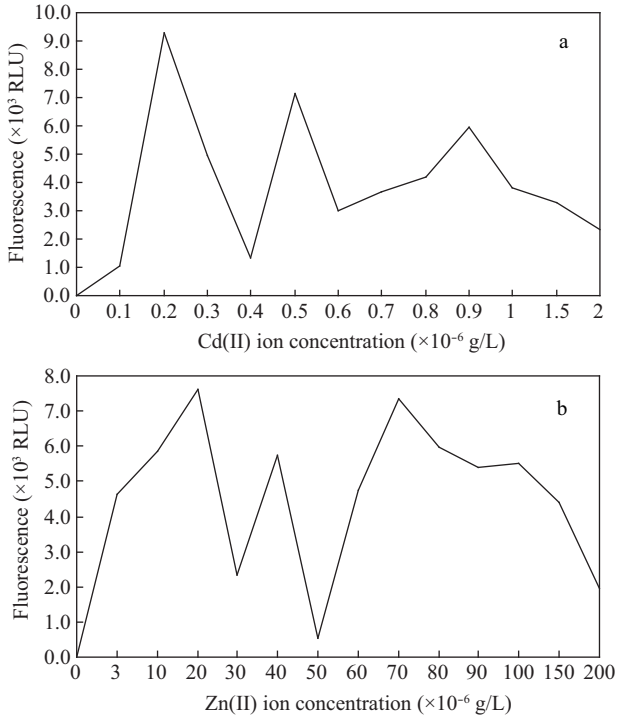

Fig. 5 Absolute fluorescence of the *E. coli* AC-Hg biosensor at various concentrations of inducing Zn(II) and Cd(II) ions.

density) decline steeply beyond 0.5 μmol/L concentration of $HgCl_2$ (Babai and Ron, 1998).

2.4 Specificity of the *E. coli* AC-Hg biosensor

The *E. coli* AC-Hg biosensor was induced with Zn(II) and Cd(II) ions in concentration ranges (3–200) × 10^{-6} and (0.1–2) × 10^{-6} g/L, respectively. The absolute fluorescence values (Y) recorded were 10^3 times lower than those obtained with Hg(II), indicating that the biosensor is highly specific for Hg(II) ions (Fig. 5).

2.5 Mathematical model for estimating Hg(II) ion concentration

Since both the fluorescence and cell density of the *E. coli* AC-Hg biosensor are linearly related to Hg(II) ion concentration, a formula was derived by multiple linear regression of absolute fluorescence value ('*A*' measured in RLU) and cell density (OD_{600}) data. Equation (2) can be used to estimate Hg(II) ions ($C_{100–1700\ nmol/L}$) in unknown water samples within the range 100–1700 nmol/L with 96% accuracy (R^2 = 0.958).

$$C_{100–1700\ nmol/L} = -2540.5854 + 0.0001877A + 2290.27102 \times OD_{600} \quad (2)$$

The accuracy of Eq. (2) was established by testing standard water samples prepared in the lab containing Hg(II) concentrations other than the ones used to derive it.

3 Conclusions

The biosensor constructed in this study is highly stable at very high concentrations of Hg(II) ions and can be used

to detect and quantify bioavailable Hg(II) ions in polluted water samples. Moreover, integration of the biosensor cassette into the *E. coli* genome greatly reduces the chances of plasmid loss and gene flow to other bacteria in the event of an accidental introduction to the nature. The regression model developed for the estimation is also suitable for field applications since it does not require elaborate treatment of input data. This biosensor can be made more user friendly for field use by developing it into a portable field kit for easier handling and transport.

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