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Designing a bacterial biosensor for detection of mercury in water solutions

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Abstract: Due to increasing advances in physical and chemical techniques for the assessment of pollutants in the environment, there is an immediate demand for a bioassay that can report both the presence of an analyte and its biological effects. In accordance with this need, there has been a fast growth in whole-cell biosensor technology. In this study we aimed to design a whole-cell bacterial biosensor to detect mercury in liquid solutions. The *Pseudomonas* pBS228 *merR* gene and its related promoter/operator was synthesized by Bioneer. The green fluorescence protein (*GFP*) gene was used as a reporter. *GFP* was cloned downstream of the *merR* gene. The construct, including the merR promoter, gene, and *GFP*, was cloned in a pUC19 vector and transferred into *E. coli* BL21 (DE3) bacteria. Transformed bacteria were used as whole-cell biosensors for detecting mercury. Mercury detection was monitored by means of microscopy and fluorometry techniques. Transformed BL21 (DE3) biosensors responded mainly to Hg(II), with the lowest detectable concentration being 10^{-8} M during a 3-h exposure induction period. Our results demonstrated that the noninfectious bacterial biosensors developed in the present study could be beneficial and enforceable in detection of mercury in contaminated water samples at concentrations as low as 10^{-8} M.

Key words: *merR*, mercury, green fluorescent proteins, whole cell biosensors, biosensing techniques

1. Introduction

Environmental pollution is becoming a major problem; designing a proper remediation tool requires an understanding about the pollutant source and the available means of its detection (McElroy and Green, 1955; Turner, 2000; Belkin, 2003). Several common methods are used for detecting analytes in environments. The conventional one is highly sensitive and accurate and is based on physical and chemical analysis. However, reference chemical laboratories and a complete set of arrays of analytical experiments are necessary for such an extensive and costly analysis. A second approach is the use of biosensors, such as transgenic bacterial cells, that are able to detect the pollutant in complex environments. These reporter biosystems, produced by means of genetic engineering tools, can be used to detect and even analyze chemical compounds and pollutants such as mercury (Kohler et al., 2000; Alonso et al., 2003; Rothert et al., 2005; Jeong et al., 2007; Amaro et al., 2011).

According to the World Health Organization (WHO), the maximum allowable amount of mercury in surface and drinking waters is less than 0.2 µg/L, which is much lower than standard concentrations of other metals in these waters (WHO, 1997). Mercury poisoning can lead to several diseases, including acrodynia ('pink disease'), Hunter–Russell syndrome, and Minamata disease (Bjørklund, 1995). Bacterial biosensors, especially whole cells, have opened a new field in development of methods for easy, cheap, quick, and user-friendly detection of mercury and other metals in the environment (Farré and Barceló, 2009; Liu et al., 2010; Rantala et al., 2011). In this study, we designed and evaluated a whole-cell biosensor for the detection of mercury in a solution. The recombinant plasmid construct was named pUMERG and was developed by insertion of the green fluorescence protein (*GFP*) gene downstream of the *merR* gene, which is controlled by the merR promoter.

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2. Materials and methods

2.1. Plasmid construction

All the molecular methods were performed according to Sambrook et al. (1989). *E. coli* strains and plasmids constructed and used in this study, along with their abbreviations and descriptions, are listed in the Table. The O/P region and the entire *merR* gene were amplified by polymerase chain reaction (PCR) from a commercial plasmid containing the *merR* gene from *Pseudomonas* pBS228. Oligonucleotide primers used for PCRs were designed based on the nucleotide sequence of *Pseudomonas* pBS228 deposited at GenBank with the accession number NC_008357.1. *EcoRl* and *BamH*I restriction endonuclease cut sites were incorporated at both ends of the sequence. *Pfu* DNA polymerase (Fermentas, USA) was used in PCRs. The resulting 540-bp fragment was digested with *EcoRl* and *BamH*I enzymes and ligated with T4 DNA ligase (Fermentas) into pUC19 (previously digested with the same enzymes). The obtained 3.21-kb construct (pUMER) was transformed into *E. coli* DH5α (replicating host) and *E. coli* BL21 (DE3) (expressing host).

PIVEX-GFP was used as the PCR template to amplify the *GFP* gene. The resulting PCR fragment was ligated to *BamHl* and *Pstl* sites next to *merR* in pUMER after predigestions with *BamHl* and *Pstl* enzymes. The final 3.96-kb construct was transformed into the *E. coli* strains DH5α and BL21 (DE3) (Ahmad et al., 2014).

2.2. Mercury assay conditions

E. coli strains were grown in Luria-Bertani (LB) medium at 37 °C in an orbital incubator (BioTek, USA). *E. coli* strains carrying plasmids constructed from pUC19 were selected with ampicillin (100 μg/mL). For mercury sensitivity tests, culturing in test tubes began with 100-fold dilutions of fresh overnight culture of *E.coli* strains harboring pUMERG in 5 mL of medium. To assess cell growth, a spectrophotometer (OPTIZEN, Poland) was used for measuring the absorbance at 600 nm (A_{600}). When the A_{600} of a culture reached 0.4–0.6, cells were diluted 100-fold in LB broth, and 0.1 mL of these dilutions was spread on LB agar plates that contained 100 μg/mL ampicillin and various concentrations of mercury ions. Plates were incubated overnight at 25–30 °C. The colonies of bacterial cells were counted after 18 h (Stocker et al., 2003).

2.3. Microscopy

To evaluate the function of the developed biosensor, one colony of *E. coli* strain BL21 (DE3) harboring pUMERG was grown overnight in LB medium supplemented with 100 μg/mL of ampicillin at 37 °C. The overnight culture was diluted 100-fold in fresh LB medium supplemented with 100 μg/mL of ampicillin and incubated at 37 °C in an orbital shaker at 220 rpm until the optical density at 600 nm reached 0.6. Various concentrations of mercury ions (10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M, and 10^{-4} M) were added to the bacterial cultures.

Assay mixtures contained 2 mL of diluted cell suspension and 2 mL of fresh LB medium containing Hg(II). Cultures were incubated at 30 °C in a rotary shaker (190–220 rpm). For GFP measurements, culture samples of 200 μL (after 3 h) were centrifuged for 2 min at 15,000 × *g* and the supernatant was decanted. The cell pellet was

Table. Plasmids and *E. coli* strains constructed and used in this study.

Plasmid or strain	Designation or genotype	Description	Source
Plasmids			
pUC19		Basic vector, amp^R gene	Fermentas
pIVEX-GFP		Source of *gfp* gene, amp^R gene	Pasteur Institute
pGEM T – easy		Source of *merR* gene and related operator/promoter	Bioneer
pUMER	pUC19:P_{mrer}-merR	*merR* expression from P_{mer} by mercury induction	This study
pUMERG	pUC19:P_{mer}-merR-gfp	*merR* and *gfp* expression from P_{mer} by mercury induction	This study
Strains			
E. coli DH5α	F- *gyrA96* (Nalr) *recA1 relA1 endA1 thi-1 hsdR17* (rk–mk +) *glnV44 deoR* Δ*(lacZYA-argF) U169* [φ80dΔ*(lacZ)M15*]	Replicating host	Razi Institute
E. coli BL21 (DE3)	F- *ompT hsdSB(rB- mB-) gal dcm (DE3)*	Expression host	Cinnagen

washed 2 times with 500 µL of phosphate-buffered saline (PBS) and resuspended in an appropriate amount of PBS (between 10 and 50 µL, depending on the amount of cells) (Stocker et al., 2003; Tani et al., 2009).

The specific amount of cell suspension used for microscopy and the fluorescence of individual cells was characterized by fluorescence microscopy (Liao and Ou, 2005). *E. coli* strain BL21 (DE3), which was harboring pIVEX-GFP, was used as the positive control and was induced by IPTG. *E. coli* BL21 (DE3) with plasmid pUC19 was used as a negative control. Cells were viewed using an epifluorescent microscope (Zeiss, Germany) equipped with a mercury lamp (100 W), an excitation filter (385–425 nm), a dichroic mirror (450 nm long pass), and an emission filter (500–540 nm) (Tani et al., 2009).

2.4. Fluorometry

The expression activity of the biosensor cells was assessed by the evaluation of the GFP fluorescence of cells grown in LB medium containing different concentrations of mercury ions. Cell growth was measured by spectrophotometry (optical density at 600 nm). A fluorometer (JASCO FP-6200; JASCO, USA) was used for measuring the fluorescence produced by biosensor cells that were grown in culture. The excitation and the emission wavelength of the fluorometer was set at 490/10 nm and 530/10 nm. *E. coli* BL21 carrying pUC19 was used as the baseline sample to zero the instrument.

We considered the influence of cell growth on the intensity of signal and noise. The entire intensities of fluorescence (arbitrary fluorescence units, AFU) were divided by the A_{600} of subcultures without subtracting or any background values. Noise and signal were explained as AFU/A_{600} and AFU in the presence or absence of Hg ions, respectively. The signal-to-noise ratios were defined as the ratio of signal to noise (AFU/A_{600}) (Tani et al., 2009).

Crude fluorescence amounts were measured by the instrument's arbitrary relative fluorescent units (AFUs). Specific fluorescence intensity is the crude fluorescence's intensity, measured in relative fluorescence units, separated by the optical density at 600 nm assessed at each time point. Several measurements were obtained for each sample after 3 h of exposure (Tani et al., 2009).

3. Results

3.1. Fluorometry

Fluorescent intensity was measured in different concentrations of mercury over 3 h and the data was used for drawing a standard curve (Figure 1). At mercury concentrations of less than 10^{-8} M, expression of *GFP* was very low and we observed no fluorescent emission. This could be due to *merR* binding to the related operator/promoter, which suppresses the transcription and translation of *GFP*. In higher concentrations of mercury ions (10^{-4} M), *GFP* expression was lowered by saturation of *merR* with mercury ions and the cytotoxic effects of these ions on bacterial cells. The mercury detection rate was measured between 10^{-8} to 10^{-4} M.

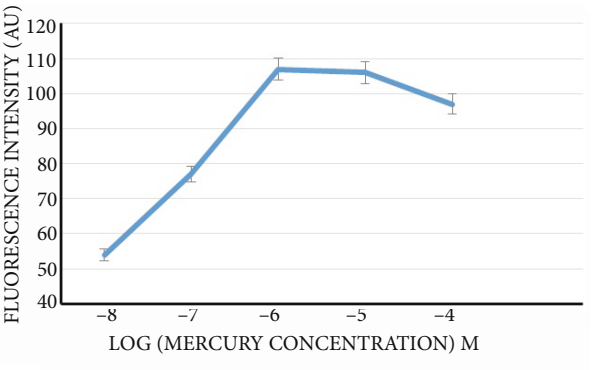

Figure 1. The curve of concentration-dependent mercury ions, obtained by measuring the fluorescence intensity in a 3-h period. Data are expressed as mean ± one standard deviation.

3.2. Florescence microscopy

The florescent emission induced by mercury was observed by florescence microscopy (Figure 2).

A direct relationship was seen between mercury concentrations and fluorescent emission. Low concentrations of mercury could only induce transcription of *GFP* in a small number of bacteria.

4. Discussion

Due to the increasing environmental pollution caused by heavy metals and related compounds, there is an urgent need to develop advanced analytical tools and methods for rapid and easy detection of these types of contaminates in the environment. Heavy metals, including lead, mercury, copper, cadmium, nickel, and chromium, are important factors for human health and environmental pollution. Contamination with these metals causes serious health problems, and thus fast and simple detection methods are vital for both human health and environmental protection. According to studies, mercury contamination of the groundwater and rivers in industrial cities like Ahvaz, Mahshahr, Arak, Isfahan, and other cities in Iran is much higher than the standard range. In recent years, several technologies have been developed with the aim of detecting or removing these contaminants. Among these technologies, those based on the use of microorganisms as biological sensors were highly regarded. The biologic biosensor provides several advantages compared to other conventional methods.

The developed biosensor is highly accessible and provides a more feasible and economic approach. The biosensor is easy to work with in situ and can be used in the

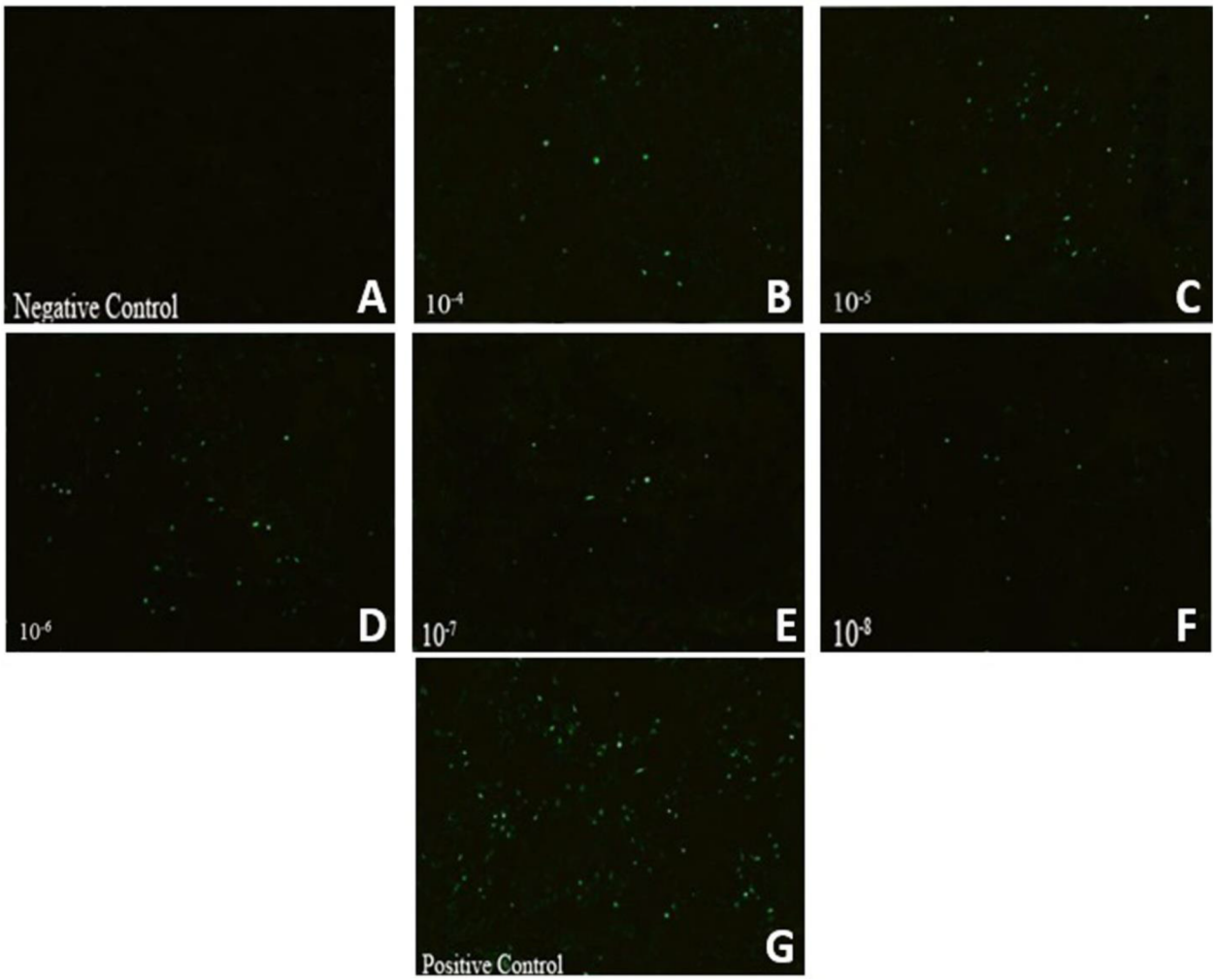

Figure 2. Fluorescence produced by biosensors after exposure to different concentrations of mercury. **(A)** Negative control, **(B)** 10^{-4} M, **(C)** 10^{-5} M, **(D)** 10^{-6} M, **(E)** 10^{-7} M, **(F)** 10^{-8} M, and **(G)** positive control.

field for mercury detection. The results can be quantified without use of a specific instrument. The biosensor can be easily regenerated and propagated for further studies. However, the main advantage of this biosensor is its ability to analyze biologic samples and in vivo application to detect trace amounts of mercury.

Although biosensor technologies around the world have remarkable advances, these technologies in Iran are at the beginning of their path. In Iran there are very limited reports related to the production or use of biosensors, especially whole-cell biosensors, for the detection of pollution. In this study, we have used the *merR* gene (from *Pseudomonas* pBS228), pUC19 plasmid, and *GFP* reporter gene to develop a whole-cell biosensor capable of detecting mercury in aquatic samples. Based on our knowledge, there are not any reports of building such a construct for biologically detecting mercury. There are whole-cell biosensors that use reporter systems and upstream mercury-inducible promoters (Ivask et al., 2001; Aguilera et al., 2006; Nagata et al., 2010). There are extensive reports of using bacterial biosensors since 1993; over time, the specificity and sensitivity of these biosensors have increased. For example, several biosensors were designed using reporter genes *luxAB*, *GFP*, and *LacZYA* by Hansen and Sørensen (2000), with the purpose of detecting mercury compounds. These biosensors have a sensitivity lower than biosensors designed by Goddard (2009). Sensitivity of biosensors produced in the past decade were in the micromolar range, while sensitivities of newly designed biosensors are in the nano- and picomolar ranges. Perhaps the development of genetic engineering techniques and the use of more sensitive reporter genes are the reasons for this increased sensitivity. In order to construct a whole-cell bacterial biosensor to detect mercury, we were very interested in using the pBS228 plasmid of *Pseudomonas* as the source of

the *merR* gene and its related operator/promoter. Bacteria use special proteins to negate the effects of heavy metals on their biological systems. These proteins are usually under the control of substrate-inducible promoters. The mercury resistance operon encodes several proteins, including merP and merT for transporting the mercury ions into cells, merB for lysing the bonds between methyl and mercury in methylmercury, and merA for the reduction and neutralization of mercury (Nucifora et al., 1989). Expression of these genes is under the control of a repressor/activator protein, which is encoded by another gene called *merR*. In the presence of mercury ions, the repressive role of this protein, located on the operation part of the other genes, converts to an active role and other genes in the operon are expressed. Through bioinformatic analyses, we focused on *merR* genes and MerR proteins from plasmid MS 145 and *Pseudomonas* pBS228.

Our bioinformatic analysis showed a high level of similarity (85.5%) between these two merR proteins, but in comparative view it can easily be observed that there are three cysteine residues in the helix-turn-helix motif of merR from pBS228, while one of them is absent in the *merR* gene of MS 145. Since merR proteins have a helix-turn-helix motif, it is not surprising that different amino acid compositions result in different sensitivities (Rosinski and Atchley, 1999; Gajiwala and Burley, 2000). Obviously, more practical analysis, such as directed substitution mutations, is necessary to evaluate the importance of each amino acid residue in the protein's function. *GFP* was selected as a reporter gene due to the ease of examining gene expression, sensitivity, ability to evaluate quality, stability, and absence of natural disturbances. Due to these advantages, as well as ease of access to this reporter gene, *GFP* was chosen for this study. The *GFP* gene was extracted from pIVEX-GFP plasmid. Two controls were used in this study: *E. coli* (DE3) transformed with pUC19 as a negative control and *E. coli* (DE3) bearing pIVEX-GFP as a positive control. The obtained data from our developed biosensor were evaluated using these controls. As can be observed in Figures 1 and 2, there is a direct relationship between increased concentrations of mercury ions and the number of cells expressing the *GFP* gene. Biosensor cells designed in this study were capable of detecting concentrations of mercury ions in a range of 10^{-7} and 10^{-8} M. According to the standard values of mercury in drinking water, this detection range is consistent with the proposed application of the biosensor. Due to the lack of the mercury-neutralizing *merA* gene in our biosensor cell, contrary to expectations, a significant reduction in the number and intensity of fluorescent bacteria was observed at high concentrations of mercury (10^{-4}) (Figure 1). One of the most valuable benefits of these biosensors is detecting the bioavailable part of mercury in samples. In the presence of mercury ions, bacterial biosensors lead to signals that could be easily and in a very short time recognized and measured. Therefore, these systems are known as real-time diagnostic systems. Although our biosensor cannot perform as powerfully as advanced mercury biosensors, this simple biosensor is able to respond to our needs in the fields of metal contamination.

The sensitivity and short response time of the developed biosensor could be advantageous in in situ detection of mercury contamination of drinking water and environmental fields. Furthermore, the obtained data can be easily interpreted by an inexperienced person.

It is hoped that further studies on the capabilities of this biosensor and enhancement of its accuracy will lead to its production on an industrial scale so that, in addition to water, it will be able to detect the exact quality and quantity of mercury in various samples, such as bodily fluids. Further improvements can be applied to the whole-cell detection system and may include mercury elimination features.

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