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Construction of green fluorescent protein based bacterial biosensor for heavy metal remediation

C. Edward Raja; *G. S. Selvam

Department of Biochemistry, School of Biological Sciences, Center for Excellence in Genomic Sciences, Madurai Kamaraj University, Madurai 625021, India

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ABSTRACT: Environmental contamination by heavy metals is a worldwide problem. Therefore, it is necessary to develop sensitive, effective and inexpensive methods, which can efficiently monitor and determine the level of hazardous metals in the environment. Conventional techniques to analyze metals, suffer from the disadvantages of high cost. Alternatively, development of simple system for monitoring heavy metals pollution is therefore needed. The present approach is based on the use of bacteria that are genetically engineered so that a measurable signal is produced when the bacteria are in contact with the bioavailable metal ions. Reporter genes are widely used as genetic tools for quantification and detection of specific cell population, gene expression and constructing whole cell biosensors as specific and sensitive devices for measuring biologically relevant concentrations of pollutants. An attempt has been made to construct the reporter gene enhanced green fluorescent protein and was expressed under the control of *cadR* gene, responsible for cadmium resistance. Recombinant strain *Escherichia coli* cadR30 was used, that carried *cadR* gene in pET30b expression vector and cloned. Clones confirmed by the expression of enhanced green fluorescent protein was detected under ultraviolet illumination and separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The construction of green fluorescent protein based *Escherichia coli* bacterial biosensor was developed based on green fluorescent protein expression under the control *cadR* gene of *Pseudomonas aeruginosa* BC15. The constructed bacterial biosensor is useful and applicable in determining the availability of heavy metals in soil and wastewater.

Keywords: *Aquatic environment; Contamination; E.coli; Pseudomonas aeruginosa; Reporter gene*

INTRODUCTION

The pollution of the environment with toxic heavy metals is spreading through the world along with industrial progress. Cadmium (Cd), lead (Pb) and other heavy metals are of considerable interest due to their toxicity and extensive application in the industry (Gueu *et al.,* 2007; Karbassi *et al.,* 2008). Therefore, it is necessary to develop sensitive, effective and inexpensive methods which can efficiently monitor and determine the presence and amount of hazardous metals in the environment. Traditionally, the environmental risk caused by heavy metals pollution is determined by quantification of total metals after digestion with strong acids by using conventional analytical methods (Liao *et al.,* 2006). Conventional techniques to analyze metals include chemical precipitation, ion exchange, chelation, membrane separation (Samarghandi *et al.,* 2007; Malakootian *et al.,* 2009) and cold vapour atomic adsorption spectrometry, inductively coupled plasma mass spectrometry, UV visible spectrometry and X-ray absorption spectroscopy (APHA, 1992; Townsend, 1998). These techniques are highly precise, though suffering from disadvantages of high costs (Mulchandani and Bassi, 1995; D'Souza, 2001). Therefore, development of a simple alternate system for monitoring heavy metals by microorganisms, due to its potential applications in environmental protection and toxic heavy metals recovery, is essential (Andreazza *et al.,* 2010). Biosensor is an analytical device that consists of immobilized biological material in intimate contact with a compatible transducer, which will convert the biochemical signal into a quantifiable electrical signal (Gronow, 1984; Chien and Shih, 2007). The biosensors rely on analysis of gene expression typically by creating transcriptional fusions between a promoter of interest and the reporter gene expression serves as a measure of the availability of specific pollutants in complex environments (Liu *et al.,* 2004; Nouri *et al.,* 2009; 2011). The major attributes of a good biosensing system are

*Corresponding Author Email: *drselvamgsbiochem@rediffmail.com*
Tel.:+914522459123; Fax: +91452 2459181

its specificity, reliability, ability to function in optically opaque solution, real-time analysis and simplicity of operation (D'Souza, 2001). Bacterial biosensors are microorganisms engineered to detect target chemical compounds or changes in physico chemical conditions through inducible expression of reporter protein (Vandeer meer, 2004). Several metal specific bacterial sensors for the detection of bioavailable metals were developed with fusing metal regulatory protein gene with various reporter genes by many authors (Selifonova *et al.,* 1993; Tauriainen *et al.,* 1998; Ivask *et al.,* 2001). Bacterial biosensors for Cd and Pb have been previously described utilizing reporter genes such as *lacZ*, *lux* and *luc* in the transcription fusion constructs (Tauriainen *et al.,* 1998; Shetty *et al.,* 2003). Green fluorescent protein (GFP) is an auto illuminating protein isolated from the jellyfish *Aequorea victoria* (Chalfie *et al.,* 1994) is increasingly being used as a reporter gene. GFP is the reporter whose autofluorescence can be produced without the addition of an exogenous substrate or adenosinetriphosphate (ATP) (Stiner *et al.,* 2002). GFP has been used extensively to measure gene expression in identified transplanted cells and analyze differentiation processes in mammalian systems (Yagi, 2007). In the present study, an attempt was made to construct GFP-based bacterial biosensor *E. coli* expressed under the control of *cadR* gene of *Pseudomonas aeruginosa* BC15. This research was carried out from January to June 2008 at the Department of Biochemistry, School of Biological Sciences, Madurai Kamaraj University, Madurai, South India.

MATERIALS AND METHODS

The source and growth conditions of the strains were used in the study is mentioned in Table 1. Plasmid DNA from bacterial strains was isolated by alkaline lysis method. The plasmid DNA was used to transform *Escherichia coli* (*E. coli)* BL21 by calcium chloride transformation method. The plasmid DNA isolation, transformation procedure adopted was as described by Sambrook *et al.* (1989). The vector map of pEGFP-1 is shown in Fig. 1. It is a selectable vector for monitoring promoter activity in mammalian cells via fluorescence of a GFP derivative. The vector backbone contains pUC-derived *ori* for propagation in *E. coli*.

The plasmid DNA pEGFP-1 was isolated and digested with *Bam*HI / *Not*I and resulting fragment EGFP was reintroduced into *Bam*HI / *Not*I digested pET30b and then transformed to *E. coli* BL21. Transformants were selected on Luria-Bertani (LB) agar plates containing

Table 1: Bacterial strains and plasmids used in this study

Strain / plasmid	Relevant phenotype / genotype	Source / reference
Pseudomonas aeruginosa strain BC15	Heavy metal resistance	Edward Raja and Selvam, 2009
E. coli DH5α	supE44lacU169(Φ80lacZM15) hsdR17recA1endA1gyrA96thi-1relA	Invitrogen (CA,USA)
BL21(DE3)	hsdS gal (λcIts857ind1 Sam7nin5lacUV5-T7gene1)	Novagen (CA,USA)
pET30b	Expression vector	
E. coli cadR30	*E. coli* carrying *cadR*	Novagen (CA,USA)
		P. aeruginosa BC15

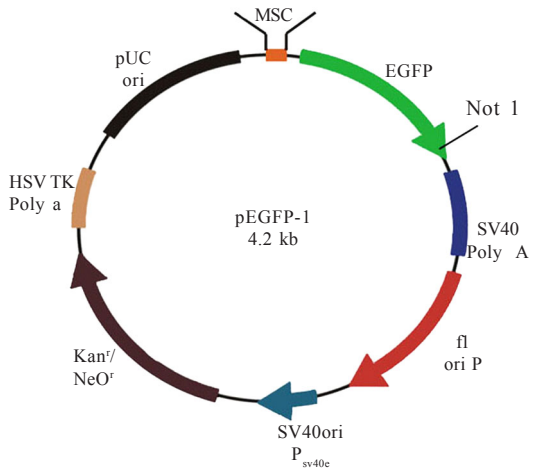

Fig. 1: Vector map of pEGFP-1

kanamycin (30 µg/mL) and IPTG (40 mg/mL). Cultures of *E. coli* BL21 (DE3) cells carrying *E. coli*EGFP4 was grown overnight in LB medium supplemented with 30 µg/mL of kanamycin. After 12 h, 1 % inoculum was sub cultured in fresh LB medium and incubated at 37 °C with vigorous shaking. When the culture reached 0.7 Optical density (OD), the cells were induced with 40 mg/mL Isopropyl β-D-1-thiogalactopyranoside (IPTG) and growth was continued for 5 h. Cells were harvested by centrifugation for 10 min at 12,000 rpm at 4 °C and pellet was suspended in 2x Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) lysis buffer. It was then heated in a water bath at 95 °C for 2 min. The lysate were harvested by centrifugation for 20 min at 10,000 rpm. The protein samples were analyzed in SDS-PAGE by the method of Laemmli (1970).

The *cadR* gene was amplified by using specific

primers *CadR F* (5'-CGATGGGAT CCGGAGGGTT GGCATGAAGATCGG-3') and *CadR* R (5'-CGGAATTCCTATGCACGAACTGGTCGCGC-3')which contains *Bam*HI and *Eco*RI restriction sites respectively. A Polymerase chain reaction (PCR) was performed with a 50 µL reaction mixture containing 10 ng of DNA extract as the template with each primer at a concentration of 0.5 mM, 1.5 mM Magnesium chloride ($MgCl_2$) and each Deoxynucleoside triphosphates (dNTPs) at a concentration of 50 mM, as well as 1units of *Taq* polymerase. After the initial denaturation for 5 min at 94 °C, denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, extension at 72 °C for 30 sec for 2 cycles, then next two cycles denaturation at 94 °C for 1 min, annealing at 63 °C for 1 min and extension at 72 °C for 30 sec, finally 31 cycles carried out denaturation at 94 °C, annealing at 61 °C for 1 min, and extension at 72 °C for 30 sec and then a final extension step consisting of 5 min at 72 °C. PCR was carried out in a Gene AMP PCR system 2700. PCR products were analyzed in 1.5 % agarose gel electrophoresis. The plasmid *E. coli*cadR30 containing *cadR* gene was amplified by PCR reaction. The amplified product was digested with *Bam*HI and *Eco*RI and then reintroduced into *Bam*HI and *Eco*RI digested *E. coli*EGFP4. After ligation, recombinants were transformed into *E. coli* BL21. The colonies were selected on LB agar plates supplemented with appropriate antibiotics.

RESULTS AND DISCUSSION

The GFP was obtained from the jellyfish *Aequorea Victoria,* and GFP gene was transferred, expressed in a wide range of organisms like mammals (Ludin *et al.,* 1996), fishes (Moss *et al.,* 1996), insects (Wang and Hazelrigg, 1994), plants (Casper and Holt, 1996), yeasts (Niedenthal *et al.,* 1996) and a broad variety of bacteria (Chalfi *et al.,* 1994). GFP has become a popular reporter for gene activity in bacteria. Therefore, it is generally being used either to establish the conditional expression of a gene in response to a specific substance, growth condition, or habitat (Barker *et al.,* 1998; Shetty *et al.,* 1999). The heavy metals such as Cd, Pb, nickel (Ni), zinc (Zn) and chromium (Cr) represent a serious problem to the environment and public health when they contaminate superficial and ground water (Barros *et al.,* 2008). Cd is extremely toxic even in low concentrations, which tend to bioaccumulate in organisms and ecosystems (Kopera *et al.,* 2004; Giaginis *et al.,* 2006). Inhaling cadmium loaded dust quickly leads to respiratory tract, kidney problems and ingestion of any significant amount of cadmium causes immediate poisoning and inducing the damage to liver and kidney (Il'yasova and Schwartz, 2005). Whole cell biosensors utilizing microorganisms tackle and overcome several concerns raised with other conventional methods, because they are usually cheap, simple nature and easy to maintain while offering a sensitive response to the toxicity of a sample (Gu *et al.,* 2004). In the present study, GFP was isolated from mammalian expression vector EGFP-1 and identified plasmid size was 4.2 kilo base pairs (kb) (Fig. 2a). The plasmid pEGFP-1 was digested with *Bam*HI / *Not*I and then 800 base pairs (bp) released fragment EGFP (Fig. 2b) was reintroduced into *Bam*HI / *Not*I digest pET30b expression vector (Fig. 2c). The resulting recombinants were used to transform *E. coli* BL21. The colonies were selected based on the expression of EGFP with IPTG induction. Initially the EGFP was detected under Ultraviolet (UV) illumination and region of insert EGFP was confirmed through restriction analysis (Fig. 2d). *E. coli* expressing GFP

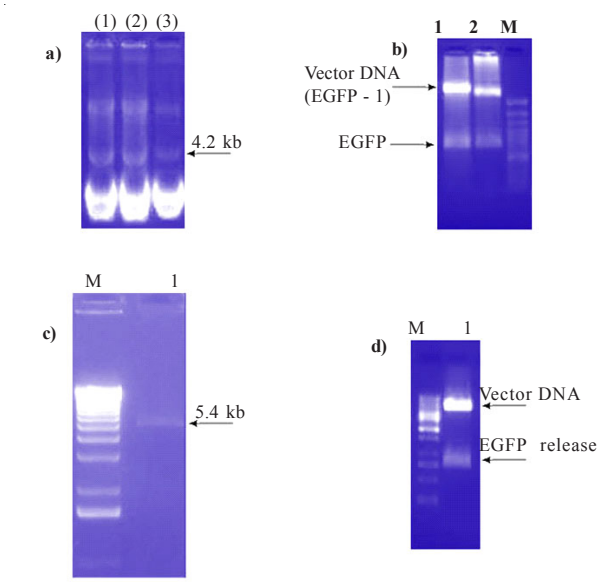

Fig. 2: (a) Isolation of EGFP-1 plasmid DNA by alkaline lysis method. Lanes 1, 2 and 3-= Plasmid DNA EGFP-1. (b) Restriction digestion of EGFP from mammalian vector. Lanes 1, 2= EFGP and vector and Lane M= Marker. (c) Restriction digestion of pET30b. Lane M= 1kb DNA ladder; Lane 1= restricted fragment of pET30b. (d) Restriction digestion of plasmid pETEGFP4. Lane M= 1 kb DNA ladder and Lane 1= Insert EGFP

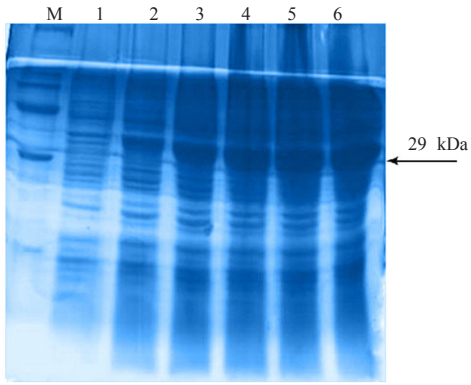

Fig. 3: Expression of GFP in *E. coli*. Lane M= Protein marker; Lane 1= Control (Without IPTG induction); Lanes 2, 3, 4, 5 and 6= Expressed GFPs in different hours

was grown in LB medium. After harvest, bacteria harbouring *E. coli*EGFP4 exhibited a substantial fluorescence.

The integrity of the recombinant GFP was analyzed on SDS-PAGE (Fig. 3). The estimated molecular weight of enhanced GFP was 29 kilo Dalton (kDa), which is in agreement with the calculated theoretical weight of 29.484 kDa for native GFP. Similar observation has been reported earlier (Prachayasittikul *et al.,* 2001). Generally reporter genes are widely used as genetic tools for quantification and detection of specific cell population, gene expression and root colonisation in complex samples (Cassidy *et al.,* 2000). Novel areas for applying reporter genes in constructing whole cell biosensors as specific and sensitive devices for measuring biologically relevant concentrations of pollutants (Willardson *et al.,* 1998). GFP has been successfully used as reporter within bacterial and yeast hosts due to the ease of *in situ* detection and the minimal metabolic cost of host cells (Roberto *et al.,* 2002). In sensor bacteria, expression of a reporter gene is controlled by a metal responsive regulatory element, which usually originates from bacteria that are naturally resistant to a particular heavy metal. The regulatory element can be coupled to a reporter gene through a gene fusion that upon expression produces a readily measurable signal in response to a particular metal. Thus in the presence of particular heavy metals, the amount of reporter protein inside the cell increases. Hence, the amount of given metal was detected by measuring the reported protein produced by the sensor bacteria (Liao *et al.,* 2006).

The plasmid DNA was isolated from *E. colicadR*30 by alkaline lysis method. The plasmid *E. colicadR30* containing *cadR* gene (528 bp) was amplified and confirmed in 1.5 % agarose gel electrophoresis (Fig. 4). Recombinant plasmids were constructed from *Bam*HI / *Not*I digested *E. coli* EGFP4 and *Bam*HI / *Not*I cut *cadR* region carrying plasmid *E. coli cadR*30. The construction of bacterial biosensor is shown in Fig. 5a. Out of 200 transformants, four colonies were selected based on the expression of GFP. The selected colonies were designated as *E.coli* cadREGFP4, *cadR*EGFP14, *cadR*EGFP29 and *cadR*EGFP36. The biosensor plasmids adopted with EGFP were detected initially under UV illumination (Fig. 5b) and presence of *cadR* region was confirmed by PCR reaction (Fig. 5c). GFP-based *E. coli cadR*EGFP14 was developed based on the GFP expression under the control of *cadR* gene of *P. aeruginosa* BC15 to be applicable for heavy metal bioremediation. In the same manner, GFP-based biosensor was developed from *Staphylococcus aureus* plasmid p1258 (Liao *et al.,* 2006). A sensor plasmid was constructed by inserting the regulation unit from the *cadA* determinant of plasmid pI258 to control the expression of firefly luciferase. The sensor plasmid pTOO24 was capable of replicating in Gram negative bacteria. The expression of the reporter gene as a function of added extracellular heavy metals was studied in *Staphylococcus aureus* strain RN4220 and *Bacillus subtilis* strain BR151. The developed sensor strains RN4220 (pTOO24) and BR 151(pT0024) mainly respond to cadmium, lead and antimony, respectively (Tauriainen *et al.,* 1998). The bacterial biosensor that responds to Pb by expressing GFP was constructed by the genetic element that senses Pb includes the regulatory protein gene (PbrR) along with operator / promoter (PbrO/P)

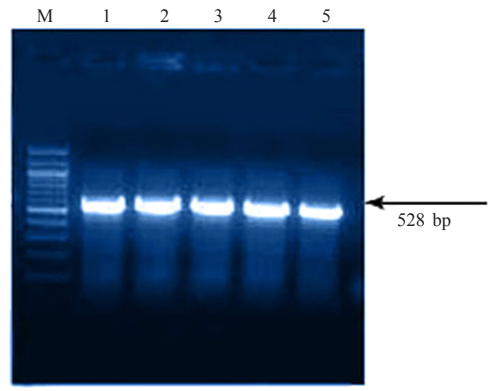

Fig. 4: Amplification of *cadR*. Lane M= 100 bp ladder and Lanes 1, 2, 3, 4, and 5= PCR products.

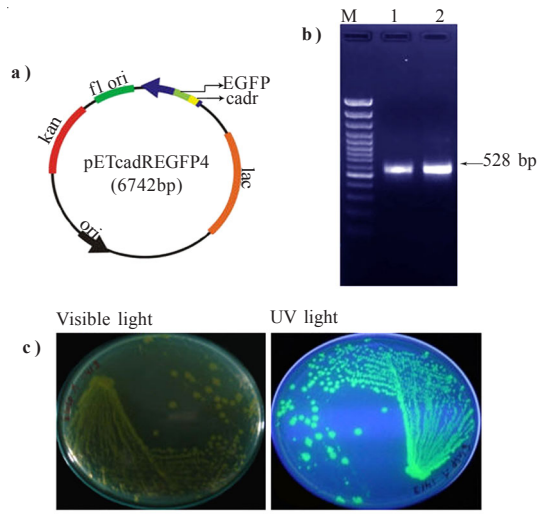

Fig. 5: (a) Cloning and construction of biosensor plasmid. (b) Amplification of *cadR* in *E. coli* cadR30EGFP4 by PCR reaction. Lane M= 100 bp ladder and Lanes 1 and 2= PCR amplified products. (c) EGFP expression in biosensor plasmid under visible and UV light

of the Pb resistance operon from plasmid pMOL30 (Chakraborty *et al.*, 2008). The present results demonstrate that the EGFP could successfully be used as a biosensor to determine the bioavailability of heavy metals in contaminated sediment, soil and wastewater.

CONCLUSION

The biosensor assays are sensitive, fast, inexpensive and less laborious, thus ideal for screening the presence of heavy metals in contaminated environment. The bacterial hosts incorporating the GFP-based sensor have proven to be a rapid and sensitive reporter system for the detection and monitoring of pollutants in environmental samples in developed countries. Therefore, the development of prokaryotic biosensors for local application would, in addition, also provide a cost-effective method of detecting environmental pollutants. The future aspect of the study is to develop GFP-based assays that would be appropriate and cost effective for environmental applications, to compare the GFP-based assay with standard water testing methods, and to develop local skills in GFP-based assays.

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AUTHOR (S) BIOSKETCHES

Edward Raja, C., M.Sc., Ph.D., Department of Biochemistry, Centre for Excellence in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai-625 021, India. Email: *edwardrajac@gmail.com*

Selvam, G. S., M.Sc., M. Phil., Ph.D., Full professor, Department of Biochemistry, Centre for Excellence in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai-625 021, India. Email: *drselvamgsbiochem@rediffmail.com*

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