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Dr. K.G. Hiremath

Head Department of Botany
Dhempe College of Arts & Science
Miramar, Panaji
Goa–403 001

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Detection and Measurement of Ultra-trace Levels of Mercury and Copper by Genetically Engineered Microbial Sensors

S.K. Dubey*, David S. Holmes, G.R. Rao and S. Gangolli

INTRODUCTION

Many traditional analytical methods such as atomic absorption spectrometry (AAS), atomic emission spectrometry (AES), mass spectrometry (MS) and high pressure liquid chromatography (HPLC) have been used to measure metals, metalloids and organic compounds (Beaty, 1988) but these analytical methods involve time-consuming and often destructive sample preparation and require highly expensive and sophisticated instruments. Though they enable precise quantitation of total chemical or ion concentration, they fail to provide information on what fraction of the total metal is in free form and hence bioavailable. This type of information, vital in developing bioremediation and biodegradation strategies, can readily be obtained by the use of biosensors.

What are biosensors? A biosensor is a detection or measuring device containing a biological component which senses and responds to a target analyte in a quantifiable manner. In recent years, extensive research work has appeared on the use of a wide-range of biological components which provide the molecular recognition element for the biosensor (Rechnitz, 1988). These biological components include chemoreceptors, antibodies/antigens and biocatalysts such as isolated enzymes, DNA sequences, metabolites, whole cells (microorganisms), plant and animal tissues. Holmes and his associates have developed several bioluminescent biosensors that are

* Laboratory of Bacterial Genetics and Environmental Biotechnology Department of Microbiology, Goa University, Taleigao Plateau, Goa-403 206, India.

based on the ability of genetically engineered bacteria to emit visible light in response to target analytes viz. Cu, Hg, PCB (Holmes et.al. 1993a,b; Gangolli et.al. 1993; Dubey et.al. 1993). Biosensors have got unique attributes such as their sensitivity, and specificity to the target analyte. Such attractive characteristics have made these biosensors an important tool in pharmaceutical and other biotech industries involved in production of health care products, food processing and environmental monitoring of toxic substances.

In last two decades use of lux genes as a bioreporter has received immense attention and popularity due to their important and valuable attributes as compared to traditional gene reporters such as CAT, LacZ, GUS, etc. (Burlage et.al. 1990; Corbisier and Silver, 1992; Holmes et.al. 1993a, b; Heitzer et.al. 1994; Loessner et.al. 1996; Iizumi et.al. 1998; Wahlstrom and Saris, 1999).

There is a pressing demand for a simple and sensitive analytical technique to detect bioavailable levels of metals such as Hg, Cd, Cu, Zn, As, Pb, Sb and organometals viz. Methyl Hg, Tetra-ethyl Lead (TEL), tributyl-tins (TBTs) directly which is present in the aquatic environment. Development of bioluminsecent microbial sensors for biomonitoring of such environmental pollutants present at ultratrace levels have proved to be an important ground breaking research in the field of environmental biotechnology and genetic engineering.

In order to accomplish this work, it is of vital importance to understand the mechanisms that microbial cells employ to regulate metal responsive operons and hence metal ion resistance. A greater understanding of metal ion resistance mechanism has made it possible to customize biosensors using several different genetic regulatory elements specifically responsive to those analytes viz. metals, organo-metals and metalloids.

It is interesting to note that microorganisms specifically bacteria respresent a wide genetic diversity and possess metal resistance determinants either on chromosomal genome or on plasmids or transposons (Ohtake et al. 1987; Silver and Misra, 1988; Cervantes and Ohtake, 1988; Cervantes and Silver, 1992; Lebrun et.al. 1994; Silver and Phung, 1996).

The mercury and copper biosensors developed, offer many advantages: their exquisite specificity to the target metal, high sensitivity, easy detection and measurement. The presence of a metal (Hg or Cu) is readily revealed by the bioluminescence produced by the biosensor microorganism. It can be seen by naked eye, measured by luminometers and also documented using CCD camera.

In this report we describe the ability of these genetically engineered microbial biosensors to detect ultratrace quantities of Hg or Cu ions which makes them ideal tools to detect these toxic metals in contaminated environmental samples, industrial effluents, sludges and clinical samples such as saliva, blood and urine.

MATERIALS AND METHODS

Plasmids and Bacterial Strains: Plasmids pDU1003 (gift from Prof. Simon Silver) and pUCD615 (gift from Prof. C. I. Kado) were used to construct the mercury biosensor. Plasmids pPA223 (gift from Prof. B.T.O. Lee) and pUCD615 were used to construct the copper biosensor. *E.coli* JM101 (Stratagene, U.S.A.) was used for transformation experiments. Transformants serve as biosensors for specific metal analytes viz. Cu and Hg.

Growth of Biosensor Cells: Biosensor cells were grown at 30°C in Luria-Bertani (LB) medium or on LB solid medium (solidified with 1.5% agar) in the presence of 100 µg/ml of ampicillin. Mercury biosensor cells grown to log phase were immobilized in LB-agar matrix to prepare

biodiscs and biofilms. These biofilms with embedded biosensor cells were also tested for their viability and analyte detection performance.

Measurements of Growth and Bioluminescence: Bacterial cell growth was measured in Spectronic 20 (Bosch & Lomb). Light emission from bacteria grown in liquid culture was recorded in a BioOrbit luminometer (Model 1250) in millivolts (Geiselhart *et al.* 1991) or in MGM Optocomp-I luminometer in relative light units (RLUs). Light emission from bacteria grown on solid media was recorded using an intensified CCD camera and an Argus-10 image processor. The images were analyzed with an LG-3 video capture board (Scion, Inc. USA) and the NIH image software (public domain) in a Macintosh II-CX computer (Fig. 1).

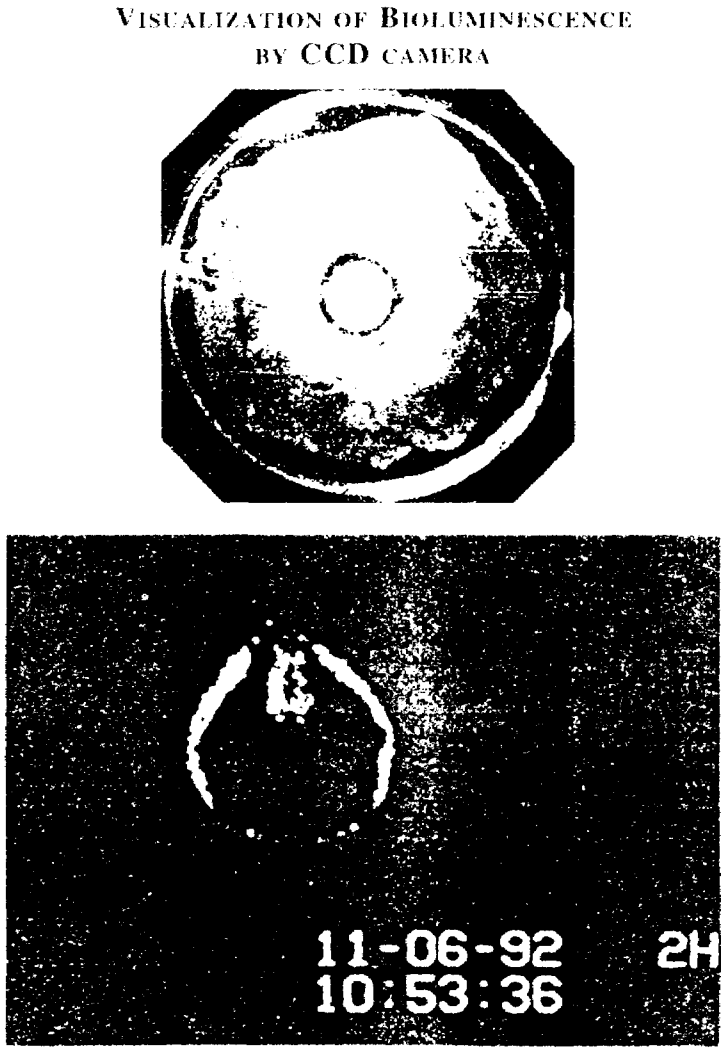


Fig. 1 **A. Visualization of bioluminescence by CCD camera.**

B. Real organizational set up of CCD camera, image intensifier, image processor, video monitor, VCR and personal computer (PC).

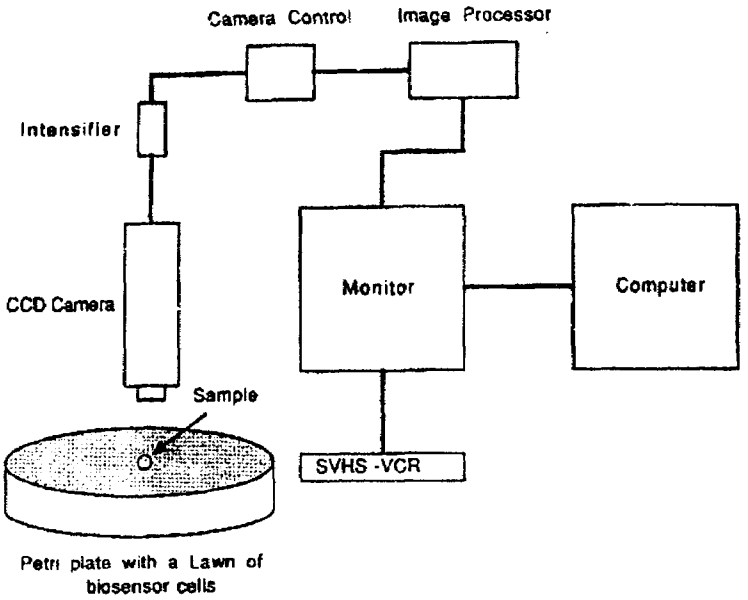


Fig. 1 C. **Diagrammatic sketch of instrumentation set up to record bioluminescence.**

RESULTS

Construction of Biosensor Organisms: Lux structural genes from *Vibrio fischeri* were fused with genetic regulatory elements from *Escherichia coli* and *Serratia marcescens* , which respond to copper and mercury ions, respectively (Fig. 2). Cells of *E.coli* JM101 were then transformed separately with these constructs. The transformants (biosensors), *E.coli* JM101/pCULUX and *E.coli* JM101/pSD30 emit light in response to copper and mercury ions, respectively. Simplified diagrammatic representation of these constructs and the products of five *lux* structural genes are shown in Fig. 2A. The biochemical mechanism of bioluminescence is also shown in Fig. 2B.

GENES AND PROTEINS INVOLVED IN METAL INDUCED LIGHT PRODUCTION

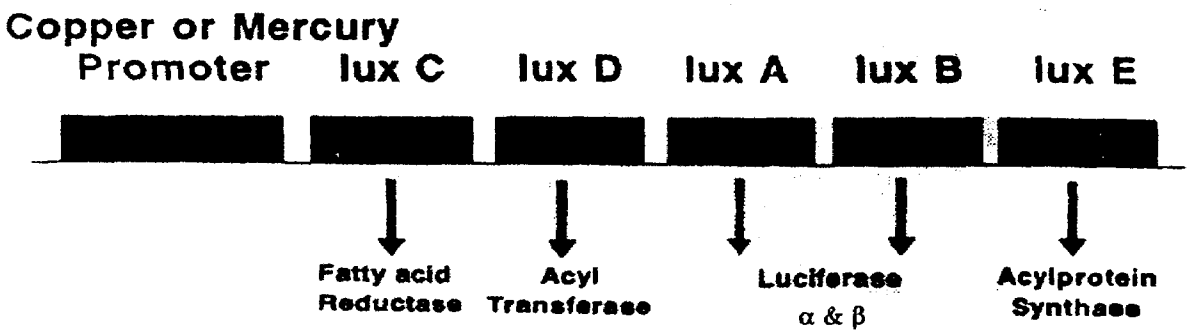


Fig. 2 A. **Diagrammatic representation of the fusion of metal responsive regulatory elements upstream of the *lux* genes. They activate the *lux* genes resulting in bioluminescence in response to Cu and Hg ions.**

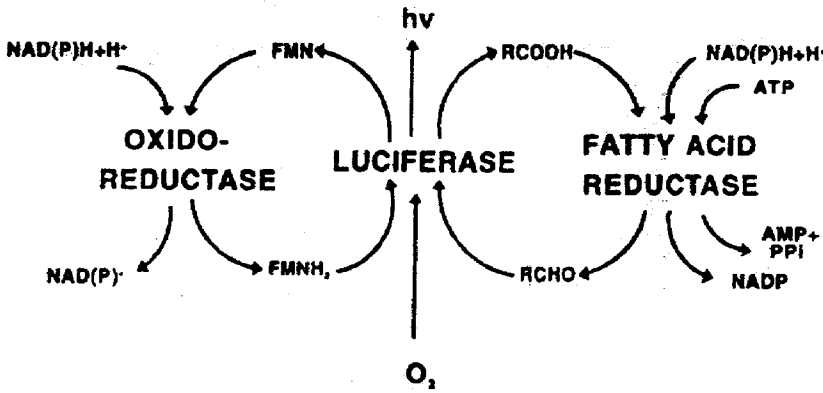


Fig. 2 B. Biochemical mechanism of bioluminescence.

Sensitivity and dynamic range of biosensors: The biosensors grown in LB broth responded to increasing concentrations of Cu and Hg. They both produced similar maximal light output. It is in the range of 10^5 to 10^6 RLUs (Relative light units) per ml of cell culture. 10^6 RLUs correspond to approximately $4x10^3$ photons/sec/cell. For both the biosensors the light output peaks in their midlog growth phase. The dynamic range, limit of detection and specificity are shown in Table 1.

Table—1 Dynamic range, limit of detection and specificity of copper and mercury biosensors@

	Cu biosensor	*Hg biosensor*
Concentration showing max. light output	1 mM	1 µm
Dynamic range	1 µM - 1 mM	0.01 nM – 1.0 µM
Lowest detection limit	1 nmol/ml	0.01 pmol/ml*
Specificity to:		
Target metal - Cu	>95.5%	0
Target metal - Hg	0	100%
Related cations (0.1 mM)		
Cobalt	0.5%	0
Cadmium	0	0
Nickel	0	0
Zinc	0	0

@ Data based on luminescence measured in BioOrbit luminometer.

* Using MGM Optocomp -I luminometer detection of 0.2 to 2 fg/ml (tested in culutre, biodisc) was achieved (for comparison with other methods, see Table 2).

The copper biosensor responds maximally at 1 mM copper with a lowest detection limit of 1 nmole/ml (1 µM = 0.06 ppm), whereas the mercury biosensor detects the metal at concentrations several orders lower. It responds maximally at 1µM and the lowest detection limit is 0.01 pmole/ml (2 ppt) (Table 2). Concentration of mercury above 1µM has proved lethal for biosensor microorganism since it lacks full mer 'A' gene responsible for mercury detoxification mediated by mercuric reductase.

Table—2 Comparison of detection limits of various physical and microbial methods for mercury

Method	*Level of detection (amount/L)*	*References*
Physical		
ICP-AES	10-20 µg (10-20 ppb)	Molders (1990)
Graphite- AAS	1-5 µg (1-5 ppb)	Molders (1990)
Hydrid- AAS	0.5 µg (0.5 ppb)	Molders (1990)
ICP-MS	0.1 µg (0.1 ppb)	Molders (1990)
MCA-90-AAS	0.01 µg (10 ppt)	Moffett (1992)
Cold vapor-AAS	0.001 µg (1 ppt)	Molders (1990)
Mercury biosensors		
E. coli C600 (pGL4)	0.2 µg (200 ppt)	Molders (1990)
E. coli JM101 (pSD 30) (biodisc)	2.0 pg (.002 ppt)	Present study
E. coli JM101 (pSD 30) (biofilm)	2.0 pg (.002 ppt)	Present study
E. coli JM101 (pSD 30) (culture)	0.2 pg (.0002 ppt)	Present study

ICP-AES: Inductively coupled Plasma-Atomic Emission Spectrometry

ICP-MS: Inductively coupled Plasma-Mass Spectrometry

AAS: Atomic Absorption Spectrometry

MCA: Mercury Concentration Accessory

Specificity of Biosensors: The mercury biosensor emitted virtually no light in presence of metals like Cd, Co, Ni, Zn and Cu. The background emission of light by the copper biosensor in the absence of added copper is only 0.5% of the fully activated level. The low background emission is probably due to the uptake of trace quantities of Cu contaminating the reagent grade chemicals used in preparing LB medium. In presence of Cd^{2+}, Ni^{2+}, Zn^{2+}, and Co^{2+} ions, the copper biosensor emits 0.5-1% of the level of light induced by Cu^{2+} (Table 1). Its response to Cu^{2+} and Cu^{1+} is almost the same.

Applicability of Biosensors: Both copper and mercury biosensors are very specific and sensitive to their target analytes. The copper biosensor can detect even copper ions released from penny and also from malachite ore (Fig. 3). In addition it can also detect trace amounts of copper

VISUALIZING RELEASE OF COPPER

Penny Visualized in it's own light:

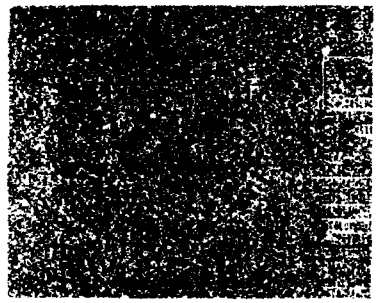


Fig. 3 A. Detection of copper released from a penny using copper biosensor.

Light from Malachite ore.

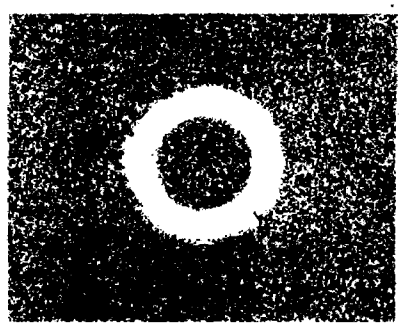


Fig. 3 B. Detection of copper released from copper ore, malachite Petri plates were prepared with 20 ml of Luria-Bertani agar and surface-inoculated with 100 µl of overnight grown culture of *E.coli* JM101/pCulux. A piece of malachite ore or a penny was placed in the centre of the agar plate. The plates were then incubated at 30°C.

ions present in mine tailings (Fig. 4). The mercury biosensor even responds to soil contaminated with mercury (Fig. 4).

The biosensor cells were grown in LB agar and poured into petri plates to develop biofilms and biodiscs. By direct spotting of $HgCl_2$ on these plates trace quantities of mercury (down to 0.5mg) can be detected. The biodiscs and biofilms prepared from LB agar embeded biosensor cells can detect as low as 2 fg Hg^{+2} /ml when the induced bioluminescence was measured in a highly sensitive Optocomp-1 luminometer. The sensitivity is further increased by 10-fold by studying the effect of mercury on growing cultures. The detection limits achieved by mercury biosensor cells and those by atomic absorption method are compared (Table 2). The mercury biosensor cells also detect ultratrace level of mercury present in water, saliva of dental patients and mercury released from dental fillings (Figs. 5 and 6).

DISCUSSION

Though there are several reports of non-*lux* reporter genes, but in few cases only *lux* genes have been used to develop specific biosensors to detect specific metal analytes (Holmes et.al.

Detection of Copper and Mercury from Environment

Detection of Mercury in Soil

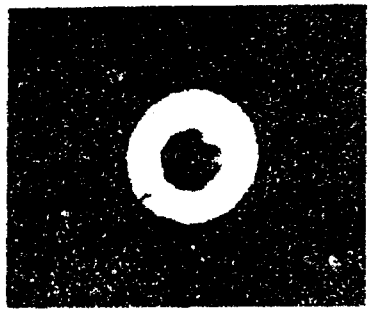


Fig. 4 A. Detection of mercury in soil sample by mercury biosensor.

Detection of Copper in Mine Water

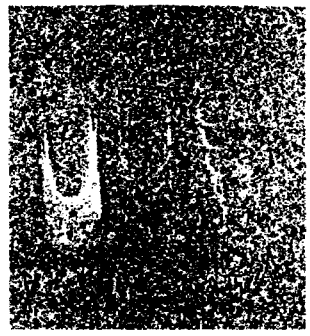


Fig. 4 B. Detection of copper in mine tailing (water) by copper biosensor.

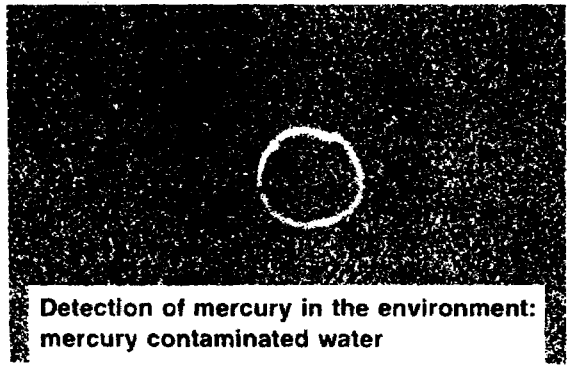


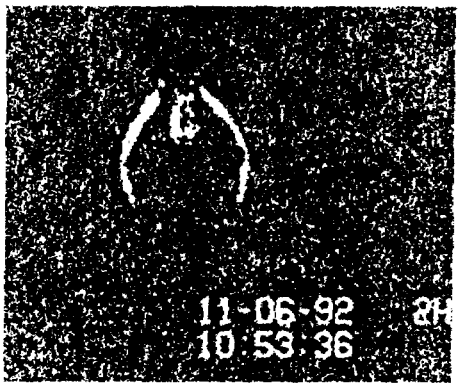
Fig. 5 Detection of mercury in mercury contaminated water sample by mercury biosensor *E.coli* JMlOl/pSD30. LB agar plates were surface-inoculated with the biosensor microoganism, and sample was placed in the centre of agar plate with lawn of biosensor cells. Light is seen as a halo around the test material.

1993a). The biosensors reported here can detect mercury and copper ions in the concentration ranges that are useful for environmental monitoring. Besides, they appear to be sensitive and specific for their target metals and do not suffer interference from relatively high concentrations of potentially competing metal ions. Their specificity may be attributed to their construction strategy since metal responsive genetic determinants (genes) are fused upstream to the *lux* reporter genes. This is accomplished by transcriptional fusion technology.

The ease of detection of light by naked eye and its quantitation by luminometers makes this type of biosensors especially valuable and attractive. They could be effectively used to monitor metal ions in a non-destructive fashion and almost in real time. The method also permits multiple or continuous image recording on a single sample over a period of time through a CCD camera.

VISUALIZATION OF MERCURY IN DENTAL SAMPLES

Extracted tooth with fillings



Saliva of a person with dental filling

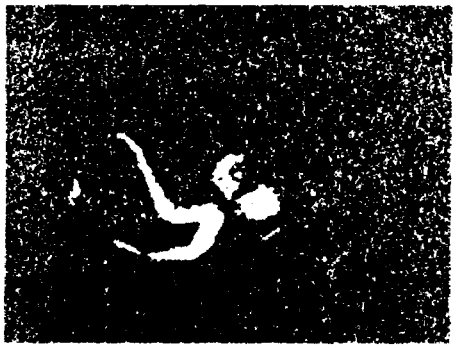


Fig. 6 **Detection of trace quantities of mercury by mercury biosensor *E.coli* JM101/pSD30 in dental amalgam (A) and saliva sample (B). Light halo was observed around dental filling showing a small inhibitory zone whereas in case of saliva sample all biosensor cells in contact with saliva were glowing.**

The bioluminescent zones captured as images are amenable for quantification, using image analysis software from NIH, U.S.A.

The traditional analytical methods do not distinguish the bioavailable component in the total chemical concentration, which is vital information for two reasons. It is this form which is responsible for the toxicity and needs bioremediation. The biosensors developed in the present study are capable of detecting the bioavailable mercury in soil, water, dental fillings (amalgam), saliva and copper released from malachite ore, penny and also in mine water.

The mercury biosensor shows exquisite specificity and responds to ultratrace levels of mercury. The lowest level of mercury detection achieved has far surpassed (by 500 to 1000-fold) the sensitivity reported for cold vapor atomic absorption spectrometry. We have successfully evaluated the viability of biofilms and biodiscs developed by immobilization of biosensor cells

in LB agar matrix. Therefore, these microbial sensors also possess immense potential for commercialization as portable device to monitor copper and mercury ions in environmental as well as clinical samples.

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