# Development of a Specific and Sensitive Bacteria Sensor for Detection of Mercury at Picomolar Levels in Environment

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A new whole-cell bacterial sensor for the detection of low concentration of mercury in environment was constructed by gene fusion between a mercury resistance (*mer*) operon from pMR26 of *Pseudomonas* strain K-62 and a promoterless *luxAB* gene from *Vibrio harveyi*. The luminescence-based biosensor was evaluated for the selectivity and sensitivity of the detection of mercury. Cadmium, lead, chromium and zinc ions did not interfere with the assay even at same concentration compared to $Hg^{2+}$. Methylmercury, phenylmercury and mercuric sulfide also did not affect the biosensor. These results reveal that the specificity of the construct is restricted to bioavailable $Hg^{2+}$. The sensitivity of the biosensor was improved by decreasing the cell density in the bioassay in addition to genetically expressing an $Hg^{2+}$ transport system which was expected to increase the amount of *mer* operon-inducing mercury in the cytoplasm. In optimized assay conditions, the lowest detectable concentration of $Hg^{2+}$ was 2 pM with 1 ml sample. This detection limit is enough to detect this compound in many contaminated and some pristine environmental samples.

**Key words** —— bioluminescence, *merR*, *merT*, *luxAB*, mercury, mercury biosensor

## INTRODUCTION

Mercury is one of the most important environmental pollutants as a global level that has been released into environment in substantial quantities by natural events and anthropogenic activities. The effect of this pollution on the ecosystem and human health are growing concerns. To prevent the environmental mercury poisoning incidents, measuring and monitoring of mercury in the environment are necessitated. Classical methods such as atomic absorption spectrometry,[1)] cold-vapor atomic fluorescence detection[2)] and inductively coupled plasma mass spectrometry[1,3)] are widely used due to their sensitive. However, these methods need sophisticated instrumentation and highly skilled personnel, complicated sample preparation and a long measuring time period. Additionally, these physicochemical methods cannot distinguish mercurials that are available to biological systems from those that existed in the environment in a harmless inert from. Analysis of bioavailable mercury is a very important issue in environmental and human hazard assessment studies.

Biosensors can provide rapid measurement without labor-intensive and time-consuming sample preparation and also provide a promising way to assess the biologically available pollutants in the environment. Several types of bacterial sensors aimed at measuring the bioavailability of mercury have been developed to complement physicochemical analysis. Most of the bacterial mercury biosensors reported to date was constructed by fusing the mercury inducible promoter and its regulatory gene, *merR* from bacterial *mer*-operon with a promoterless luminescence *lux* gene.[4–7)] In these bacterial sensors, the *mer* promoter is activated when mercury binds to the regulatory protein MerR, and luminescence is emitted when mercury is present in the bacterial cytoplasm. Since the luminescence response is quantitative, the concentration of mercury can be determined *via* measuring the intensity of *lux*-specified luminescence which was precisely regulated by the *mer* regulatory unit responding to the given mercury. All luminescence fusions developed to date except for the *mer*-controlled production of firefly luciferase,[7)] only respond to nanomolar concentrations

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of $Hg^{2+}$. This sensitivity was sufficient to detect $Hg^{2+}$ in highly contaminated environments, but would be too low for less polluted environments. The more sensitive biosensor is urgently needed in the analysis of trace amounts of mercury in the environmental and clinical samples.

In the present study, a new whole-cell bacterial sensor was constructed for the detection of low concentrations of mercury by fusing the *merR-o/p-merT* from pMR26 of *Pseudomonas* strain K-62 with a promoterless *luxAB* gene from *Vibrio harveyi*. The sensitivity, selectivity and quantitative response to mercury of this luminescence-based biosensor were evaluated.

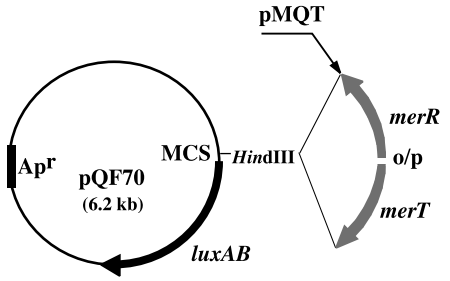


**Fig. 1.** Construction of *mer-lux* Fusion Plasmid pMQT for Detection of Mercury

Abbreviations used: $Ap^r$, gene-encoding ampicillin resistance; *R*, gene-encoding repressor/activator of the *mer* promoter; o/p, *mer* promoter/operator; *T*, gene-encoding mercury transport protein; *luxAB*, gene-encoding bacterial luciferase; MCS, multiple cloning sites.

## MATERIALS AND METHODS

**Bacterial Strain, Plasmid and Growth Conditions** —— *Escherichia coli* (*E. coli*) XL1-Blue was grown at 37°C in Luria-Bertani medium[8] and used for routine plasmid propagation. When necessary, the medium was supplemented with 100 $\mu$g/ml ampicillin. Recombinant plasmid pMRA17 carrying the broad-spectrum mercury resistance determinants (*merR-o/p-T-P-A-G-B1*) of *Pseudomonas* K-62 were used as the starting plasmid.[9] Plasmid pQF70 containing *luxAB* gene[10] was kindly supplied by M. Kropinski of Queen's University (Canada).

**Plasmid Construction** —— To construct a recombinant plasmid containing the broad-spectrum resistance *mer* operon of pMRA17, plasmid pMRA17 was digested with *Bgl*II, and the resulting 5' overhang was filled with dNTPs by DNA polymerase I (Klenow fragment). After digestion with *Eco*RV, the 4.7-kb fragment (*merR-o/p-T-P-A-G-B1*) was inserted into the *Sma*I site of cloning vector pBluescript II SK (+). The resultant plasmid was designated pMR96. Plasmid pMQT containing *merR-o/p-T* was constructed by cloning the 1.25-kb *Hind*III fragment of pMR96 into the *Hind*III site of pQF70 (Fig. 1). All restriction enzymes and other biochemical regents were purchased from Takara Shuzo Corp. (Kyoto, Japan) and Wako chemicals (Tokyo, Japan).

**Mercury Resistance Assay** —— Resistance of bacteria to mercurials was determined on petri dishes according to the method of Foster *et al*.[11] The zones of inhibition of growth around the disks were measured after incubation at 37°C for 16 hr.

**Bacterial Accumulation of Mercury** —— The mid-exponential phase *E. coli* cells carrying pMQT were suspended in Luria-Bertani medium containing 16 $\mu$M $Hg^{2+}$. After incubation at 37°C for 10 min, the amount of total mercury in the bacterial cells was analyzed.[12]

**Luminescence Measurements** —— The overnight culture of *E. coli* carrying pMQT was grown in Luria-Bertani medium containing 100 $\mu$g/ml ampicillin at 37°C to an $OD_{600}$ of 0.4. The culture was diluted to a final cell density at $5 \times 10^3$ cells/ml in the assay medium containing various concentrations of metal compounds to be tested, and incubated at 37°C for 30 min. For luciferase assay, an aliquot (1 ml) of culture was transferred a luminometric curette and the biological luminescence reaction was started by adding an aliquot (35 $\mu$l) of 10% 1-decanal. Light emission by the cloned bacteria with pMQT was monitored using a Turner Designs Luminometer (Model TD-20/20, Promega, Madison, Wisconsin, U.S.A.). The total light emitted was recorded for 10 sec counting period for each curette. Results are presented as the induction coefficients, which were defined as the relative light units (RLU) of the induced samples divided by that of the untreated sample (background luminescence). Experiments were performed in triplicate, and the standard deviation was 5 to 7% of the means.

## RESULTS AND DISCUSSION

Transcriptional fusion of a reporter gene such as *lux* or *lacZ* gene to a *mer* operon could create a bacterial strain which is able to monitor the presence of mercury by the production of quantitatively detectable reporter activity. A useful biosensor for detection of mercury requires a high sensitivity, high

**Table 1.** Mercury Uptake and Mercury Susceptibility Conferred by *mer-lux* Fusions

| Plasmid | Genotype | Uptake[a] nmol Hg/ $10^8$ cells | Inhibition zone[b] mm |
|---|---|---|---|
| pQF70 | *luxAB* | $3.4 \pm 0.15$ | $18 \pm 0.16$ |
| pMQT | *merR*-o/p-*T*-*luxAB* | $5.5 \pm 0.11$ | $28 \pm 0.33$ |

*a*) Mercury in the cells was measured after 10 min incubation with 16 $\mu$M of $HgCl_2$. *b*) Inhibition zone diameters (minus 6-mm disk diameter) with 600 nmol of $HgCl_2$. All values are means of the triplicate determinations.

specificity and quantitative response toward the compounds to be monitored. Most of the *mer-lux* biosensors constructed to date have nanomolar sensitivity.[4–7] This sensitivity was sufficient to detect mercury in highly contaminated environments, but would be too low for less polluted environments. It could be possible to enhance the sensitivity of *mer-lux* biosensor by expressing a *merT*-specified mercury transport system which was expected to increase the concentrations of the *mer* operon-inducing mercury in the cytoplasm. Because the luminescence due to the action of luciferase encoded by *lux* gene is controlled by the *mer* regulatory unit which responds quantitatively to the imported mercury in the cytoplasm.

In the present study, a new biosensor plasmid pMQT which contains *merR*, *mer* o/p, and *merT* from pMR26 of *Pseudomonas* K-62 strain[9] and a promoterless *luxAB* gene from *Vibrio harveyi*[10] for detection of low concentrations of mercury was constructed (Fig. 1). It should be important to confirm whether the luminescence emission from the recombinant bacteria with pMQT is induced by mercury, and the transcriptional gene expression is repressed by absence of mercury. The genetically engineered sensor bacteria did not emit luminescence under uninduced condition by mercury but obviously emitted luminescence under the condition of induction with 0.1 nM $Hg^{2+}$ (Data not shown). These results clearly suggest that the luminescence emission encoded by *luxAB* gene is induced when mercury is present in the cytoplasm of the recombinant bacteria.

Mercuric ion is known to be specifically transported into bacterial cells *via* MerT protein encoded by *merT* gene.[13–15] To import the inducer effectively, constitutively synthesized MerT protein was expected to enhance the sensitivity of this cellular biosensor to mercury. As shown in Table 1, the bacterium with pMQT which contains mercury-transport gene, *merT*, took up more $Hg^{2+}$ and showed more susceptibility to $Hg^{2+}$ than the bacterium with pQF70.

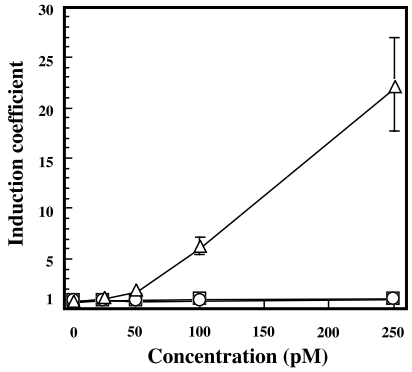


**Fig. 2.** Response of the *mer-lux* Biosensor to Mercurials

*E. coli* XL1-Blue with plasmid pMQT was incubated with various concentrations of $Hg^{2+}$ (△), $C_6H_5Hg^+$ (○) or $CH_3Hg^+$ (□). After incubation for 30 min at 37°C, the luminescence was measured as described in MATERIALS AND METHODS. All values are the means of triplicate determinations from three experiments.

These results indicate that mercury transport system encoded by *merT* is functional in the sensor bacteria. The hypersensitivity phenotype to $Hg^{2+}$ is thought to result from hyperaccumulation of toxic $Hg^{2+}$ mediated by *merT* in the absence of mercuric reductase activity. As expected, about two- to threefold elevation in luminescence emission from the recombinant bacteria carrying pMQT over the mutant with specific point mutations in the vicinal cysteine pair (Cys24 and Cys25) of *merT*, which are known to be essential for $Hg^{2+}$ transport into the bacterial cells,[15,16] in pMQT was observed at $Hg^{2+}$ concentrations of 2 to 250 pM. This result reveals that increase mercury uptake into the cells mediated by *merT* would result in activating the *mer* promoter to a greater extent, and then yielding higher titer of luminescence. The need for *mer* specific transport protein to lower the detection threshold has also been reported by Selifonava *et al*.[6]

Response of the mercury bioluminescence sensor to organomercurials and the other heavy metals was next examined. As shown in Fig. 2, no response of the sensor bacteria to the presence of the same concentrations of $CH_3Hg^+$ or $C_6H_5Hg^+$ was observed.

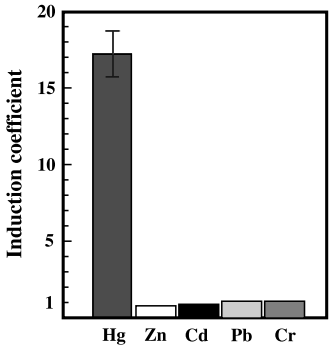


**Fig. 3.** Response of the *mer-lux* Biosensor to Heavy Metals

*E. coli* XL1-Blue with plasmid pMQT was incubated with 200 pM $Hg^{2+}$, $Cd^{2+}$, $Pb^{2+}$, $Cr^{6+}$ or $Zn^{2+}$. After incubation for 30 min at 37°C, the luminescence was measured as described in MATERIALS AND METHODS. All values are the means of triplicate determinations from three experiments.

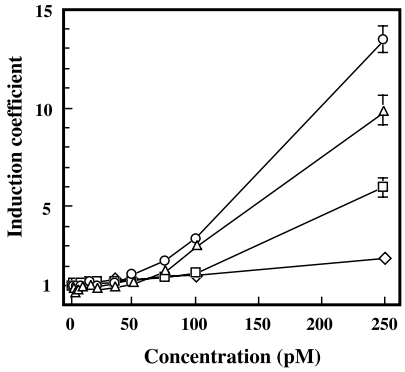


**Fig. 4.** Effect of Cell Density on the Sensitivity of the *mer-lux* Biosensor

*E. coli* XL1-Blue with plasmid pMQT was incubated with various concentrations of $Hg^{2+}$. After incubation for 30 min at 37°C, the luminescence was measured as described in MATERIALS AND METHODS. All values are the means of triplicate determinations from three experiments. △: $1 \times 10^3$ cells/ml, ○: $5 \times 10^3$ cells/ml, □; $5 \times 10^4$ cells/ml, ◇: $5 \times 10^5$ cells/ml.

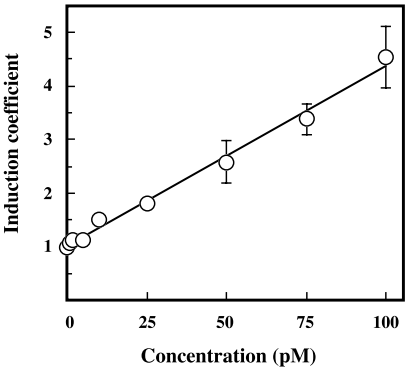


**Fig. 5.** Calibration Curves for Response of the *mer-lux* Biosensor to $Hg^{2+}$

*E. coli* XL1-Blue with plasmid pMQT ($5 \times 10^3$ cells/ml) was incubated with various concentrations of $Hg^{2+}$. After incubation for 30 min at 37°C, the luminescence was measured as described in MATERIALS AND METHODS. All values are the means of triplicate determinations from three experiments.

In addition, among the heavy metals employed, only $Hg^{2+}$ induced luminescence emission by the sensor bacteria (Fig. 3). The high specificity is thought to be due to the highly specialized and selective genetic pMR26's *merR*, a narrow spectrum regulator that has been shown to rigidly respond to $Hg^{2+}$ but not to organomercury.[9,17] The biosensor also did not respond to bio-stable mercury such as metallic mercury and mercuric sulfide (Data not shown). Therefore, the *mer-lux* biosensor developed in this study is restricted for detection of bio-affecting $Hg^{2+}$.

In addition to genetic constructs, parameters such as cell density in the assay and measurement protocols have been reported to influence the sensitivity of bioluminescence sensor.[5,18] To enhance the sensitivity of the *mer-lux* biosensor developed, the assay conditions were first optimized. The effect of cell density on the sensitivity of the *mer-lux* response was shown in Fig. 4. Luminescence production induced by the sensor bacteria was highest when $5 \times 10^3$ cells/ml were added in the assay medium at $Hg^{2+}$ concentrations below 250 pM. With our biosensor, the number of bacterial cells could be reduced to $5 \times 10^3$ cells/ml. The higher cell density above that in the assay reduced the observed luminescence level, possibly because of metal adsorption by cell wall or light adsorption due to the turbidity of the cell suspension. From these results, the cell density at $5 \times 10^3$ cells/ml is ideal for highly sensitive mercury bioluminescence sensor developed in this study for detection of low concentration of $Hg^{2+}$. The luminescence production by $5 \times 10^3$ cells/ml of the bacteria reached detectable levels within 30 min after induction with $Hg^{2+}$ concentrations below 250 pM (Data not shown). By using the optimizing assay conditions, a linear correlation was found between luminescence emission and $Hg^{2+}$ concentrations in the investigated concentration range as shown in Fig. 5. The detection threshold of this mercury bioluminescence sensor was about 2 pM. The sensitivity obtained here exceeded earlier biosensors described for mercury by Selifonova *et al.*[5] and by Tescione and Belfort.[6]

Rapid and sensitive measurements of mercury compounds are urgently required in various fields such as environment, food industry and medicine. The assay by using the biosensor developed in this

study is simple, sensitive, gives results in a relatively short time and would allow the assessment of the biologically available fraction of $Hg^{2+}$ in many contaminated and in some pristine environments.

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## REFEREN CES

1) Omang, S. H. (1971) Determination of mercury in natural waters and effluents by flameless atomic absorption spectrophotometry. *Anal. Chim. Acta*, **53**, 415–420.
2) Bloom, N. S. and Fritzgerald, W. F. (1988) Determination of volatile mercury species at the pictogram level by low-temperature gas chromatography with cold-vapor atomic fluorescence detection. *Anal. Chim. Acta*, **208**, 151–161.
3) Burlingame, A. L., Boyd, R. K. and Gaskell, S. J. (1996) Mass spectrometry. *Anal. Chem.*, **68**, 599–651.
4) Condee, C. W. and Summers, A. O. (1992) A *mer-lux* transcriptional fusion for real-time examination of in vitro gene expression kinetics and promoter response to altered superhelicity. *J. Bacteriol.*, **174**, 8094–8101.
5) Selifonova, O., Burlage, R. and Barkay, T. (1993) Bioluminescent sensors for detection of bioavailable Hg(II) in the environment. *Appl. Environ. Microbiol.*, **59**, 3083–3090.
6) Tescione, L. and Belfort, G. (1993) Construction and evaluation of a metal ion biosensor. *Biotechnol. Bioeng.*, **42**, 945–952.
7) Virta, M., Lampinen, J. and Karp, M. (1995) A luminescence-based mercury biosensor. *Proc. Anal. Chem.*, **67**, 667–669.
8) Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9) Kiyono, M., Omura, T., Inuzuka, M., Fujimori, H. and Pan-Hou, H. (1997) Nucleotide sequence and expression of the organomercurial-resistance determinants from a *Pseudomonas* K-62 plasmid, pMR26. *Gene*, **189**, 151–157.
10) Farinha, M. A. and Kropinski, A. M. (1990) Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. *J. Bacteriol.*, **172**, 3496–3499.
11) Foster, T. J., Nakahara, H., Weiss, A. A. and Silver, S. (1979) Transposon A-generated mutations in the mercuric resistance genes of plasmid R100–1. *J. Bacteriol.*, **140**, 167–181.
12) Pan-Hou, H., Hosono, M. and Imura, N. (1980) Plasmid-controlled mercury biotransformation by *Clostridium cochlearium* T-2. *Appl. Environ. Microbiol.*, **40**, 1007–1011.
13) Hobman, J. L. and Brown, N. L. (1997) Bacterial mercury-resistance genes. *Met. Ions Biol. Syst.*, **34**, 527–568.
14) Lund, P. A. and Brown, N. L. (1987) Role of *merT* and *merP* gene products of transposon Tn501 in the induction and expression of resistance to mercuric ions. *Gene*, **52**, 207–214.
15) Kiyono, M., Uno, Y., Omura, T. and Pan-Hou, H. (2000) Role of MerT and MerP from *Pseudomonas* K-62 plasmid pMR26 in the transport of phenylmercury. *Biol. Pharm. Bull.*, **23**, 279–282.
16) Morby, A. P., Hobman, J. L. and Brown, N. L. (1995) The role of cysteine residues in the transport of mercuric ions by the Tn501 MerT and MerP mercury-resistance. *Mol. Microbiol.*, **17**, 25–35.
17) Kiyono, M., Uno, Y., Omura, T. and Pan-Hou, H. (2000) Involvement of *merB* in the expression of the pMR26 *mer* operon induced by organomercurials. *J. Health Sci.*, **46**, 142–145.
18) Riether, K. B., Dollard, M. A. and Billard, P. (2001) Assessment of heavy metal bioavailability using *Escherichia coli zntAp::lux* and *copAp::lux*-based biosensors. *Appl. Microbiol. Biotechnol.*, **57**, 712–716.