Journal of Industrial Microbiology & Biotechnology (1999) 23, 668–676

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A single-use luciferase-based mercury biosensor using *Escherichia coli* HB101 immobilized in a latex copolymer film

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A single-use Hg(II) patch biosensor has been developed consisting of 1.25-cm diameter patches of two acrylic vinyl acetate copolymer layers coated on polyester. The top layer copolymer was 47 μm thick whereas the bottom layer of copolymer plus *E. coli* cells was 30 μm thick. The immobilized *E. coli* HB101 cells harbored a *mer-lux* plasmid construct and produced a detectable light signal when exposed to Hg(II). The immobilized-cell Hg(II) biosensor had a sensitivity similar to that of suspended cells but a significantly larger detection range. The levels of mercury detected by the patches ranged from 0.1 nM to 10 000 nM $HgCl_2$ in pyruvate buffer, and luciferase induction as a function of Hg(II) concentration was sigmoidal. Luciferase activity was detected in immobilized cells for more than 78 h after exposure of the cells to $HgCl_2$. Addition of 1 mM D-cysteine to the pyruvate buffer increased luciferase induction more than 100-fold in the immobilized cell patches and 3.5-fold in a comparable suspension culture. The copolymer patches with immobilized cells were stable at −20°C for at least 3 months, and the Hg(II)-induced luciferase activity after storage was similar to that of samples assayed immediately after coating. Patches stored desiccated at room temperature for 2 weeks showed lower mercury-induced luciferase activity when compared to freshly prepared patches, but they still had a considerable detection range of 1 to 10 000 nM $HgCl_2$.

Keywords: biosensor; *E. coli*; immobilization; latex film; luciferase; mercury

Introduction

Mercury contamination continues to be a public health and environmental problem. Microbial biosensors aimed at measuring the bioavailability of Hg(II) have been developed to complement chemical or physical analysis [20]. Conventional chemical detection techniques such as atomic emission spectroscopy (AES) and cold-vapor atomic fluorescence spectroscopy (CVAFS) are highly sensitive, can measure the total concentration of heavy metal contamination, but are expensive in terms of equipment and training and are complex to perform. Microorganisms that quantitatively detect toxins in the environment offer a less expensive alternative to chemical methods [15,35] and have the added advantage of detecting the biologically available fraction of the heavy metal [10].

Several types of microbial biosensors have been reported. A suspended-cell luciferase-based biosensor for detection of Hg^{2+} was developed and patented recently [32], but it requires extensive handling and preparation of the bacterial suspension for each measurement. For suspended cell assays to be reproducible, cells have to be cultivated and harvested identically each time, making these measurements difficult to perform outside a microbiology laboratory. The disadvantages of using fresh bacterial suspensions have been addressed by preparing freeze-dried cells that, upon rehydration, can measure the bioavailability of a number of heavy metals [2,7]. An alternate approach to developing biosensors, as described in this paper, is the use of viable bacteria immobilized in latex copolymer films. While alginate-immobilized *Aeromonas hydrophila* have been reported to reduce Hg^{2+} to Hg^0 in a fluidized bed reactor [21], there are no reports of using film-immobilized microorganisms as a Hg(II) indicator or biosensor.

For any viable cell biosensor to be effective, it must contain a sensitive and specific receptor linked to a reporter that is easily measurable. These requirements have been met by a number of *mer-lux* Hg(II) biosensor plasmid constructs [35,44]. The dimeric MerR/promoter complex is a very sensitive receptor ($K_m = 1 \times 10^{-8}$ to 5×10^{-8} M) [30] yet very specific for Hg(II) (for reviews of bacterial mercury resistance, see [11,24,36]). *In vitro* interference by Cd(II), Zn(II), Ag(II), Au(I), Au(III) required 1×10^2 to 1×10^3-fold higher concentrations than the inducing concentration of Hg(II) [30]. The *in vivo* interference by Cd(II) occurred at 1×10^7-fold higher concentration than the inducing concentration of Hg(II), with no *in vivo* interference observed for Zn(II), Cu(II), Mn(II) and Co(II) [44]. The bioluminescence genes (*lux*) are easily detectable in either a scintillation counter, a luminometer, a photon counting imaging device, or on photographic film [38]. The *lux* genes have been used as a reporter in numerous suspended cell systems because of the ease of non-destructive quantification, and include a naphthalene monitor [5], bacterial detection using a bacteriophage [43], and detectors for antimony [40], arsenate [6,40], cadmium [6], chromate [26], zinc [9], and waste water toxicity [12].

In this work, latex-immobilized *E. coli* HB101 cells containing different *mer-lux* fusions were tested for luciferase

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Received 5 November 1998; accepted 8 April 1999

induction by low levels of inorganic Hg(II) and compared to induction of suspended cell cultures. The results demonstrate that immobilization preserves the activity and sensitivity of *E. coli* HB101, and increases the range of Hg(II) detection possible by viable whole cells.

Materials and methods

Bacterial strains, chemicals, media, and growth conditions

E. coli strain HB101 [F$^-$*hsdR520* (r_k^-, m_B^-), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20* (Sm^r), *xyl-5*, *mtl-1*, *supE44*] was used as the host strain for all experiments. Plasmid-containing cells were grown in Antibiotic Medium 2 (Difco, Detroit, MI, USA) or in Luria-Bertani (LB) medium (10 g L^{-1} tryptone [Difco], 5 g L^{-1} yeast extract [Difco], 5 g L^{-1} NaCl [analytical grade], pH 7.2) containing 30 μg ml^{-1} kanamycin (Sigma Chemical Company, St Louis, MO, USA) at 30°C. $HgCl_2$ was purchased from JT Baker Chemical Company (Phillipsburg, NJ, USA). All other chemicals were of the highest analytic quality available.

Plasmids used are shown in Figure 1. Even though several *mer-lux* plasmid constructs have been developed [32,35,41,44], the constructs made by Selifonova and coworkers are especially useful in that each construct has been tested extensively for Hg(II) sensitivity in suspended cultures under different conditions [31,34,35]. Three plasmids, pRB28, pOS14 and pOS15, code for luciferase activity (*luxCDBE*) but differ in the subset of *mer* genes fused to the *lux* genes. pRB28 contains *merR* (the *mer* repressor/activator gene) and a truncated *merT* (one of the *mer* transporter genes) (Figure 1). A second construct, pOS14, contains *merR* and the complete set of Hg(II) transport genes (*merT*, *merP* and *merC*). The third construct, pOS15, contains *merRTPC*, the reductase gene (*merA*) and a second regulatory gene (*merD*). Induction of the *mer* operon by inorganic Hg(II) results in the production of luciferase which can be assayed by the ATP-dependent emission of photons [8].

Latex cell immobilization

Immobilization of viable *E. coli* cells using patch coating was described elsewhere [23]. *E. coli* HB101 containing pRB28, pOS14, or pOS15 were grown in 300 ml of LB medium supplemented with 30 μg ml^{-1} kanamycin at 30°C overnight in a 2-L Erlenmeyer flask at approximately

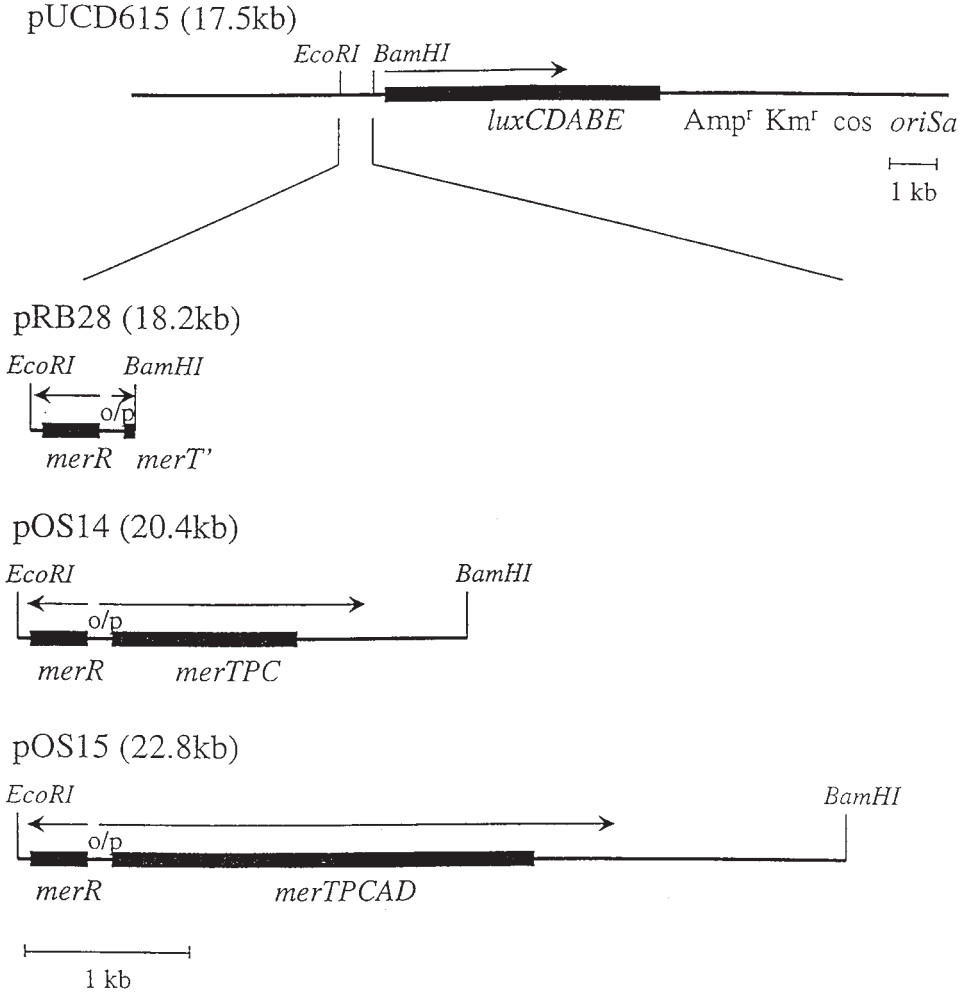

Figure 1 Plasmids pRB28, pOS14 and pOS15 derived from pUCD615. Arrows indicate the direction of transcription. Transcription of the *mer* and *lux* genes initiates from the *mer* operator/promoter region (o/p) [35].

150 rpm in a Labline shaker (model 3525CC). Cells were harvested by centrifugation for 15 min at $2800 \times g$, and the cell pellet was washed in phosphate-buffered saline (PBS) pH 7.4 [1] or pyruvate buffer (5 mM pyruvate, NaK-phosphate buffer pH 6.8 [34 mM sodium phosphate, 33 mM potassium phosphate] and 0.091 mM $(NH_4)_2SO_4$) [1]. The cell pellet was suspended in glycerol and SF091 acrylic vinyl acetate copolymer latex (Rohm and Haas, Philadelphia, PA, USA) in the ratio 1.2 g cell paste: 0.3 ml 50% (w/w) glycerol: 1 ml latex. The cell-coat mixture was coated onto a 7×9-cm polyester template using a 26-mil-wire wound rod (Mayer bar, Paul N Gardner Company, Pompano Beach, FL, USA) at 4°C. After the coated layer had dried, the template was removed, and a second layer of latex (topcoat) was coated as a sealant. The topcoat layer was dried at 4°C, and the assembly was cured at 37°C for 30 min producing patches with a dry thickness of 30 μm immobilized cell layer and 47 μm topcoat layer (Figure 2). Individual patches were excised and rehydrated in buffer. Each patch contained approximately 5×10^8 viable *E. coli* HB101 cells [23].

Induction of immobilized E. coli HB101 cells with Hg(II) and detection of luciferase activity

Latex-immobilized cells were exposed to $HgCl_2$ by soaking the patches in 10 ml of LB or pyruvate buffer containing $HgCl_2$ concentrations ranging from 0.1 to 10 000 nM in sterile glass scintillation vials. Hg(II) concentrations of the controls and low-concentration standards were confirmed by CVAFS (Brooks Rand Model III, Seattle, WA, USA). Immobilized cell samples were incubated in triplicate at 25°C, and luciferase activity was detected as counts per minute of ATP-dependent photon emission in a liquid scintillation counter (Beckman, LS 7000, Columbia, MD, USA).

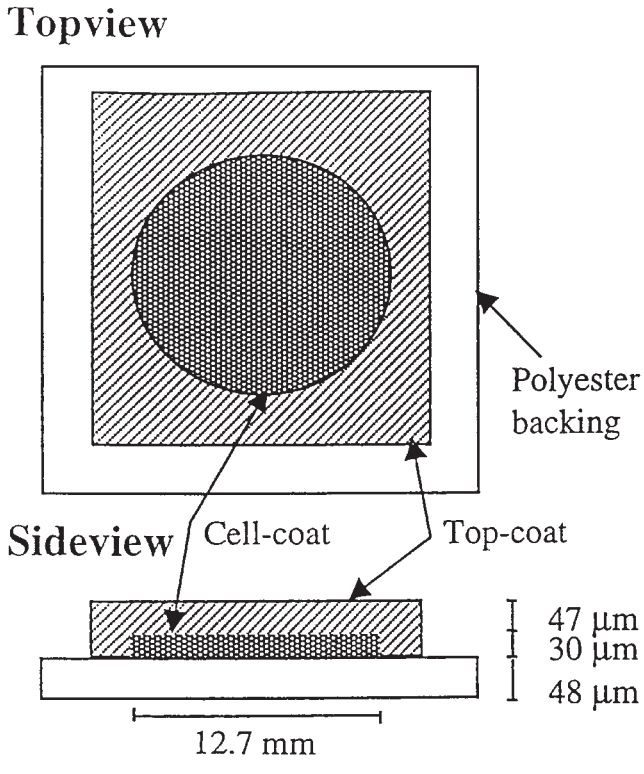

Figure 2 Immobilized mercury biosensor. Schematic of an immobilized cell patch is shown from the top view and a side view.

Induction of suspended cultures

E. coli strain HB101 harboring pRB28 was grown overnight in 50 ml LB (30°C, 250 rpm) containing 30 μg ml^{-1} kanamycin in 250-ml baffled flasks. The overnight cell suspension was pelleted by centrifugation, and the pellet was resuspended in pyruvate buffer to an OD_{660} of 0.4 which is approximately 2.2×10^8 viable cells ml^{-1}. For the assay, cells were diluted to final cell densities of 2.2×10^7 and 1×10^6 cells ml^{-1} in a total volume of 10 ml in glass scintillation vials. $HgCl_2$ concentrations for this assay ranged from 0.1 nM to 10 000 nM. Luciferase activity was measured as described for latex-immobilized cells.

Induction of E. coli HB101(pRB28) in pyruvate buffer containing D-cysteine

The effect of sulfur groups on induction of luciferase activity by $HgCl_2$ in immobilized and suspended *E. coli* strain HB101(pRB28) was studied by adding cysteine (Sigma Chemical Company) to the pyruvate buffer containing Hg(II). In this assay, various concentrations of $HgCl_2$ were preincubated with 0.1 or 1 mM cysteine for 1 h. After preincubation, an immobilized cell patch or a sample of suspended cells was added to the solution in a glass scintillation vial, and the luciferase activity was measured.

Effects of cold storage or dry storage on induction of immobilized E. coli HB101(pRB28)

Latex-immobilized cells were analyzed for storage stability at −20°C. Patches containing *E. coli* strain HB101 (pRB28) were placed in a 2-ml solution of 50% glycerol (w/w) in PBS (pH 7.4) for 3 months at −20°C. The patches were thawed and equilibrated for 6 h in PBS at ambient temperature prior to exposure to $HgCl_2$ in pyruvate buffer. To assess dry storage stability, patches were dried at room temperature with a topcoat of latex containing 25% sucrose and kept in a desiccator at room temperature for 14 days. The patches were rehydrated in PBS for 24 h prior to induction with $HgCl_2$ in pyruvate buffer. Samples not exposed to $HgCl_2$ were used as controls.

Results

Induction of luciferase in film-immobilized E. coli HB101 harboring mer-lux constructs in pyruvate buffer

Patches of *E. coli* HB101 harboring the *mer-lux* constructs were analyzed individually for luciferase activity after exposure to $HgCl_2$ at concentrations from 0.1 nM to 10 000 nM. The plasmid pRB28 contains the repressor gene *merR* and a truncated form of *merT* (designated *merT′*) [35]. Without a complete *merT*, Hg(II) enters these cells by passive transport across the cell membrane. Transcription of the *luxCDABE* genes, which are fused to *merT′*, is induced by the presence of inorganic Hg(II). Induction of luciferase activity in *E. coli* HB101 (pRB28) by 0.1, 1 or 10 nM $HgCl_2$ was not apparent during the first 5 h of incubation but increased substantially during the next 15 h after

which time the activity continued to increase or remained constant until 37 h (Figure 3a). Cells exposed to higher levels of Hg(II) had significantly different kinetics of luciferase induction. At 100, 1000 or 10 000 nM $HgCl_2$, the luciferase activity reached maximum detection levels (limited by the scintillation counter to 6.1×10^6 cpm) within the first 5 h of induction.

The plasmid pOS14 includes the complete set of transport genes *merT*, *merP* and *merC* in addition to the repressor gene *merR* and the *lux* genes, but lacks the reductase gene *merA* (Figure 1). Therefore, *E. coli* HB101 (pOS14) can actively transport Hg(II), but is unable to reduce Hg^{2+} to Hg^0. *E. coli* HB101(pOS14) in pyruvate buffer containing 0.1 nM $HgCl_2$ showed no significant luciferase activity compared to the control (Figure 3b). Hg(II) concentrations at 1 nM and 10 nM induced luciferase activity after a 4–5 h lag, and luciferase activity increased during the next 10 h of the assay. At 100 and 1000 nM $HgCl_2$, luciferase activity was evident after 2 h of incubation and reached the maximum detectable level after 5–8 h.

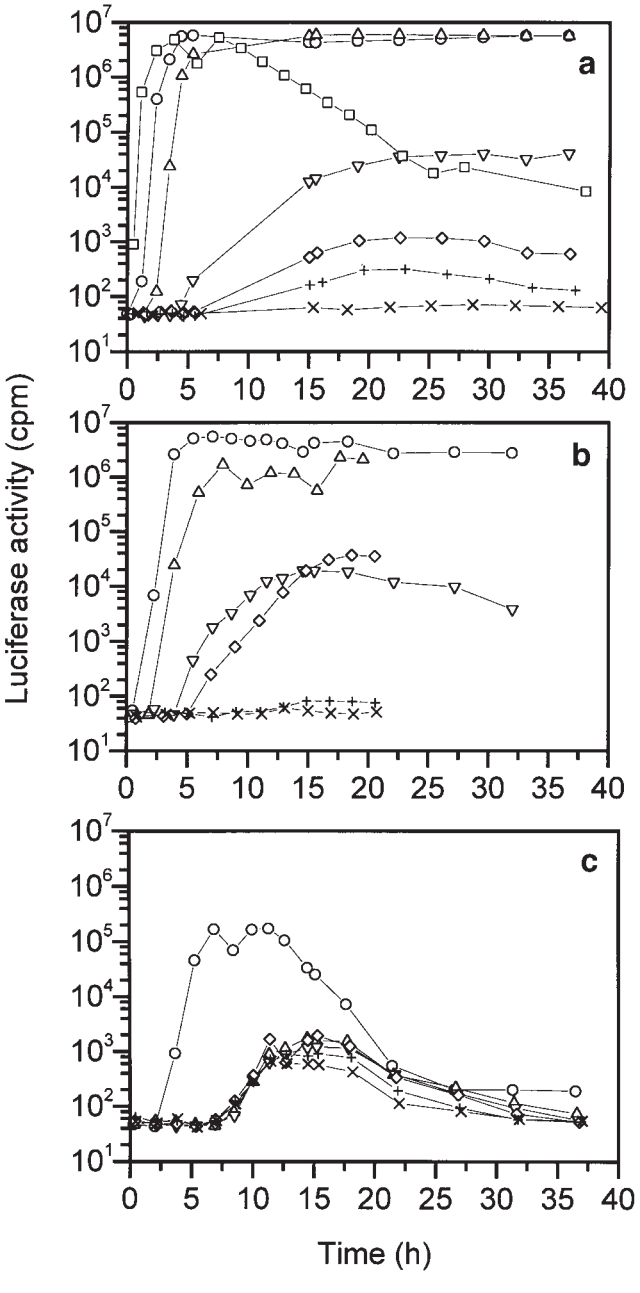

Figure 3 Luciferase activity of latex film-immobilized *E. coli* HB101 containing *mer-lux* constructs after induction by $HgCl_2$ in pyruvate buffer. (a) pRB28; (b) pOS14; (c) pOS15. Symbols: (□) 10 000 nM $HgCl_2$; (○) 1000 nM $HgCl_2$; (△) 100 nM $HgCl_2$; (▽) 10 nM $HgCl_2$; (◇) 1 nM $HgCl_2$; (+) 0.1 nM $HgCl_2$; and (×) 0 nM $HgCl_2$. Each data point represents the average of three determinations.

Plasmid pOS15 differs from pOS14 in that it includes the reductase gene *merA* and another regulatory gene, *merD*, and cells harboring pOS15 are therefore capable not only of transporting Hg^{2+} but also of reducing it to Hg^0 (Figure 1). The only concentration of Hg(II) that showed significant luciferase induction above background levels with pOS15 was 1000 nM $HgCl_2$ (Figure 3c). At this concentration, the induction of luciferase from the *mer* operon occurred within the first 2 h of exposure to $HgCl_2$, and activity reached a maximum of 2×10^5 cpm after 7 h. The response profile of pOS15 is different from the one observed for pRB28 and pOS14 in that the induced luciferase signal was lower and decreased within 5 h after reaching its maximum. The detoxification of Hg^{2+} by reduction to Hg^0 is a likely cause for the decrease, which is undesirable for a biosensor application. These results indicate that the *lux* construct in pRB28 or pOS15 is induced to the maximum detectable levels by $HgCl_2$, but induction of pRB28 displays the broadest sensitivity range.

Similar experiments were carried out with induction of the immobilized cells in LB (data not shown). The induction profiles for the three plasmids were similar to that seen with incubation in pyruvate buffer, except the lag periods before induction at lower Hg^{2+} concentrations (0.1–100 nM) were prolonged.

Induction of suspended E. coli HB101 (pRB28) in pyruvate buffer

Previous reports of Hg(II) sensitivity for *mer-lux* constructs in suspended cells have involved assays of exponentially growing cells harvested shortly before induction [31,34,35]. However, for immobilized cells, the rehydration procedure requires that the cells be kept in a stationary or non-growing state for at least 4 h prior to induction. These differences prevented direct comparison of Hg(II) sensitivity between the previously reported suspended cell assays and the immobilized cell assays. Therefore suspended cells that were in stationary phase in LB medium at least 4 h prior to induction were used for the Hg(II) induction assays. Two different cell concentrations were used (2.2×10^7 and 1.0×10^6 cells ml^{-1}) which yielded the best induction levels in the suspension cell assays [31]. When 10–1000 nM $HgCl_2$ was added to 2.2×10^7 cells ml^{-1} of HB101(pRB28), luciferase activity was detected within the first 30 min after induction (Figure 4a). At 1 nM $HgCl_2$, induction of luciferase was somewhat slower with a lag of 1 h, and maximum levels were 1000-fold lower than seen with higher $HgCl_2$ concentrations. At 10 000 and 0.1 nM $HgCl_2$, no significant luciferase activity was detected.

Suspended cultures at 1×10^6 cells ml^{-1} of HB101

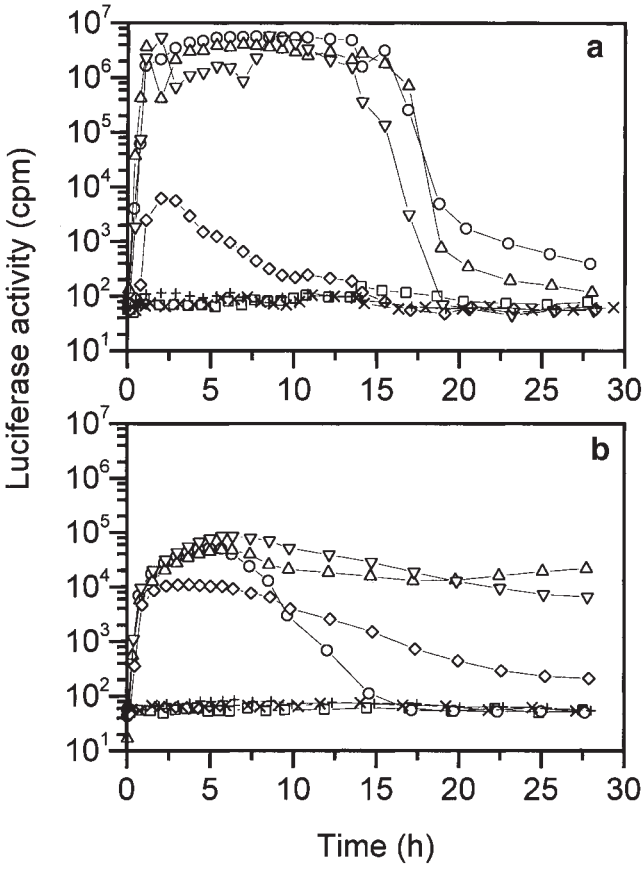

Figure 4 Luciferase activity of suspended *E. coli* HB101(pRB28) after $HgCl_2$ induction in pyruvate buffer. (a) 2.2×10^7 cells ml^{-1}; (b) 1.0×10^6 cells ml^{-1}. Symbols: (□) 10 000 nM $HgCl_2$; (○) 1000 nM $HgCl_2$; (△) 100 nM $HgCl_2$; (▽) 10 nM $HgCl_2$; (◇) 1 nM $HgCl_2$; (+) 0.1 nm $HgCl_2$; and (×) 0 nM $HgCl_2$. Each data point represents the average of three determinations.

(pRB28) showed a similar pattern of luciferase induction when compared to the more dense culture (Figure 4b). With 10–1000 nM $HgCl_2$, luciferase activity was detected within the first 30 min after induction, although the levels of luciferase activity attained were about 100-fold lower than the levels seen in cultures with 2.2×10^7 cells ml^{-1}. At 1 nM $HgCl_2$, the induced luciferase activity was similar in the two cultures. Again, there was no significant luciferase activity at 10 000 and 0.1 nM $HgCl_2$.

These results are in agreement with previous reports which showed that maximum luciferase activity for a particular culture density can be induced with lower Hg(II) concentrations as the cell concentration is lowered [31]. However, these results differ from mercury induction of luciferase activity in immobilized cells (Figure 3a). While the maximum levels of luciferase activity achieved were similar between latex-immobilized HB101 (pRB28) and the suspension cells (2.2×10^7 cells ml^{-1}), the response time was much slower (5 h *vs* 30 min) and required a different range of mercury concentrations (100–10 000 nM *vs* 10–1000 nM). No luciferase was induced in the suspension cells with 0.1 or 10 000 nM mercury. In contrast, 0.1 nM mercury induced significant amounts of luciferase and 10 000 nM mercury induced maximum levels of luciferase in the immobilized cells. Differences in response times and luciferase induction as a function of mercury concentration may reflect differences in mercury diffusion rates into suspended and immobilized cells as well as differences in tolerance to toxic levels of mercury.

Induction of latex-immobilized and suspended E. coli HB101(pRB28) with $HgCl_2$ and D-cysteine

Rasmussen and coworkers [31] reported that non-specific binding of Hg^{2+} to cells was a likely reason for the low Hg(II) sensitivity observed in high cell density experiments. Addition of a small molecule capable of binding mercury competitively and diffusing through the cell membrane may increase the availability of mercury to interact with MerR and promote gene expression. Cysteine is one candidate molecule for mercury binding and uptake. Since MerR binds mercury via three cysteine residues [14,28], molecules that bind mercury with a higher affinity than cysteine may actually deplete MerR of Hg^{2+} and effectively reduce induced gene expression. Therefore, d-cysteine was used to determine whether a carrier for Hg^{2+} would enhance induction.

Immobilized *E. coli* HB101 cells harboring pRB28 were incubated with 1 or 10 nM $HgCl_2$ in pyruvate buffer with and without 1 mM d-cysteine (Figure 5a). The maximum activity detected was 3×10^6 and 1×10^5 cpm (counts per min) for 10 and 1 nM $HgCl_2$, respectively, in the presence of 1 mM d-cysteine. In both cases, this maximum luciferase activity represented a 100-fold increase above the cells without added d-cysteine. Control samples lacking d-cysteine and Hg(II) had activities below 180 cpm, whereas control samples with d-cysteine alone had activities below 63 cpm. Immobilized cells were induced with 0.05 nM $HgCl_2$ and 1 mM d-cysteine to determine if the use of d-cysteine would increase the Hg(II) sensitivity in the assay (Figure 5a). At 0.05 nM $HgCl_2$, the maximum induction was 92 cpm, well above two standard deviations of the control containing d-cysteine without Hg(II) (61 ± 5 cpm). Therefore, the use of d-cysteine increased the Hg(II) detection sensitivity of the assay.

The dramatic increase in luciferase induction in the presence of cysteine indicates that Hg(II) as a bound thiol is taken up into the cell more efficiently compared to non-thiol bound Hg(II). To demonstrate that this effect was not specific to immobilized cells, the cysteine addition experiments were repeated for a suspension culture. Cells containing the pRB28 plasmid were harvested at least 4 h after reaching stationary phase, diluted to 1×10^6 cells ml^{-1}, and then induced with 10 nM $HgCl_2$ with or without 0.1 and 1 mM d-cysteine. In the presence of 10 nM $HgCl_2$ and 0.1 mM d-cysteine, the induction level was not significantly different from that without d-cysteine. With 1 mM d-cysteine, the maximum level of induction increased about 3.5-fold from 1.3×10^5 to 4.4×10^5 cpm when compared to the response without d-cysteine (Figure 5b).

Cysteine has been reported to induce transient amino acid starvation and the stringent response in *E. coli* by threonine deaminase inhibition [13,37], and therefore additions of threonine and isoleucine together with cysteine and Hg(II) were also studied. Induction of luciferase with 10 nM $HgCl_2$ and 1 mM d-cysteine was not affected by addition of either threonine or isoleucine to the samples

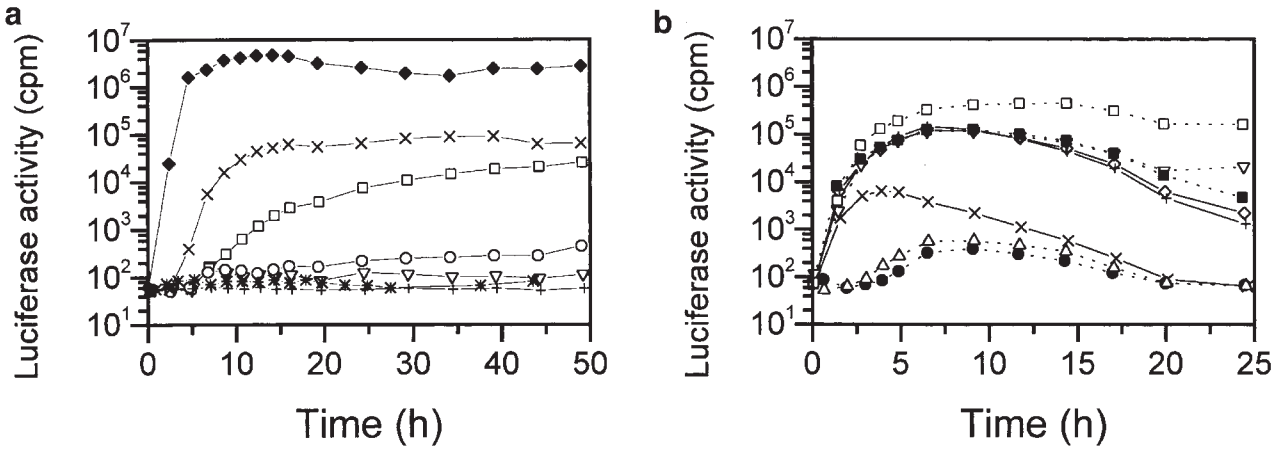

Figure 5 Effect of d-cysteine on mercury-induced luciferase activity of latex film-immobilized and suspended *E. coli* HB101 (pRB28). (a) Immobilized cells. Symbols: (◆) 10 nM $HgCl_2$ and 1 mM d-cysteine; (×) 1 nM $HgCl_2$ and 1 mM d-cysteine; (□) 10 nM $HgCl_2$ and 0 mM d-cysteine; (○) 1 nM $HgCl_2$ and 0 mM d-cysteine; (+) 0 nM $HgCl_2$ and 1 mM d-cysteine; (∗) 0.05 nM $HgCl_2$ and 1 mM d-cysteine; (▽) 0 nM $HgCl_2$ and 0 mM d-cysteine. (b) Suspended cells. Symbols: (□) 10 nM $HgCl_2$ and 1 mM d-cysteine; (▽) 10 nM $HgCl_2$ and 0.1 mM d-cysteine; (■) 10 nM $HgCl_2$; (◇) 10 nM $HgCl_2$ and 0.1 mM serine hydroxymate; (+) 10 nM $HgCl_2$ and 1 mM serine hydroxymate; (×) 10 nM $HgCl_2$ and 10 mM serine hydroxymate; (●) 0 nM $HgCl_2$; (△) 0 nM $HgCl_2$ and 1 mM d-cysteine. Each data point represents the average of three determinations.

(data not shown). To determine if the stringent response affects luciferase induction, the stringent response was induced by the addition of serine hydroxymate in the presence of Hg(II) [45]. Cells were incubated with 0.1, 1, or 10 mM serine hydroxymate and 10 nM $HgCl_2$. At 0.1 or 1 mM serine hydroxymate, there was no effect on luciferase induction with 10 mM $HgCl_2$. Addition of 10 mM serine hydroxymate decreased induction levels by about ten-fold but were still significantly higher than the uninduced controls (Figure 5b). These results indicate that enhancement of luciferase induction by cysteine is not due to amino acid starvation or induction of the stringent response.

The cysteine analog s-ethyl-cysteine was substituted for d-cysteine in an attempt to demonstrate that the increased activities observed were not d-cysteine specific and to determine whether a different sulfhydryl compound would be more effective in enhancing luciferase induction. S-ethyl-cysteine (0.1 and 1 mM) was added to suspended cell samples identical to the ones described above (data not shown). The luciferase induction observed was virtually identical to the induction seen with the same concentrations of d-cysteine, indicating that the increased induction levels due to cysteine were not d-cysteine specific.

Effects of freezing and dry storage on Hg(II) induction of immobilized E.coli HB101(pRB28)

A major disadvantage of viable cell biosensors is their short shelf-life. Maintaining the immobilized cells in a dormant but viable state by freezing or storing as a dried patch would significantly increase the usefulness of the patch biosensor. Patches containing *E. coli* HB101(pRB28) cells that were identical to those used for $HgCl_2$ induction of luciferase activity in pyruvate buffer were stored at –20°C for 3 months, then thawed and equilibrated in PBS before induction in pyruvate buffer with $HgCl_2$. The induced luciferase activity was compared to freshly prepared immobilized cell patches (Figure 6a). Freezing patches did not significantly affect luciferase induction. However, thawed patches not rehydrated before exposure to Hg(II) showed

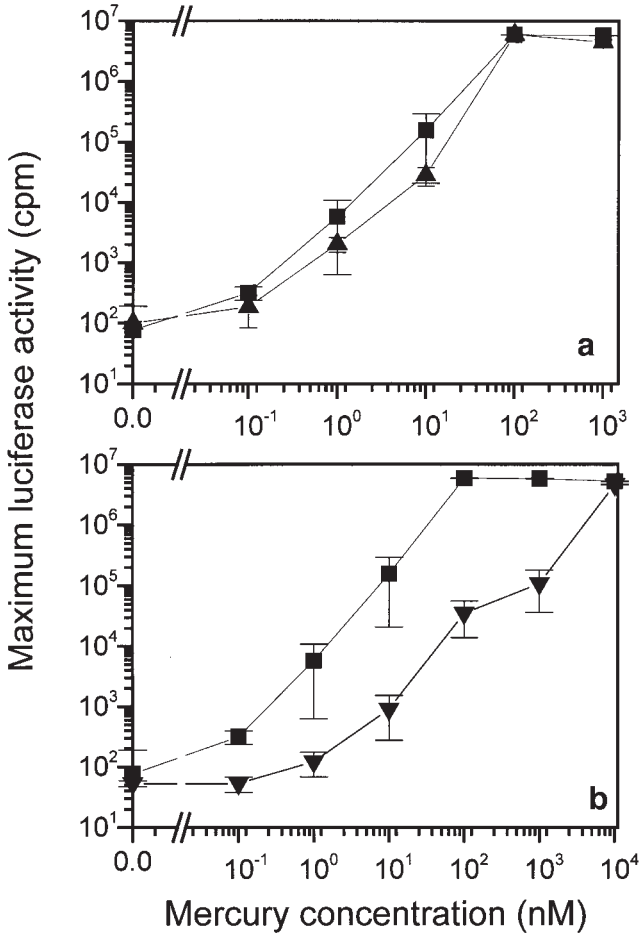

Figure 6 The effect of freezing and dry storage on latex-immobilized *E. coli* HB101(pRB28). Maximum luciferase activity was plotted as a function of mercury concentration. (a) Effect of freezing on luciferase induction: (■) freshly made immobilized cells; (▲) immobilized cells stored at –20°C for 3 months in glycerol: PBS buffer (50:50 w/w). (b) Effect of dry storage on luciferase induction: (■) freshly made immobilized cells; (▼) immobilized cells stored dry for 14 days.

no significant induction of luciferase activity (data not shown). The similarity of the induction curves indicates that the biosensor patches can be effectively stored at −20°C.

Patches were also stored in a dehydrated state to determine the effect of dry storage on Hg(II) induction of luciferase activity. After 14 days of storage in a desiccator, the patches were rehydrated in PBS and induced in pyruvate buffer with $HgCl_2$. The maximum luciferase activity achieved with the different $HgCl_2$ concentrations was generally lower than observed with freshly prepared patches, and the range of Hg(II) sensitivity was somewhat different (Figure 6b). With fresh patches, mercury concentrations between 0.1 and 100 nM could be distinguished by the luciferase induction levels, whereas with patches that had been stored dry, the sensitivity range was 1–10 000 nM $HgCl_2$. These results indicate that the biocatalytic coatings can be stored as dry patches for at least 14 days and retain their Hg(II) biosensor activity.

Discussion

The importance of mercury pollution and its effect on public health and the environment have led to the development of many highly sensitive and reproducible methods of detection (Table 1). Chemical or physical methods of Hg(II) detection are generally more sensitive than biological detection systems and can quantify the total concentration of heavy metals in a sample [3,4,17–19]. Biological detection of heavy metals has been developed to complement the more sensitive chemical and physical measurements. In spite of the lower sensitivity of biological methods compared to chemical methods for Hg(II) detection, biological detection offers low cost per sample and the ability to handle large numbers of samples simultaneously. The major advantage to the use of microorganisms to detect heavy metals is to assess the bioavailability of the metal [7]. The biosensor will detect the fraction of the total heavy metal concentration that is available to interact with the microorganism, which is critical to evaluating the potential biological impact of the contamination.

Several types of biosensors have been developed including fresh suspended cells, freeze-dried cells, and immobilized cells. Suspended cell methods require significant skill and equipment for the preparation of cell suspensions. Once the culture is made there is only minimal additional preparation needed. However, the need for constant culture preparation and the lower sensitivity have prevented the commercialization of suspended cell systems for biological detection of Hg(II). One biological detection method using suspended cells has been reported with very high Hg(II) sensitivity [44] which may surpass current analytical methods. Determinations of mercury reductase activity in bacteria from low mercury environments indicate that the *mer* operon may be active at sub-picomolar mercury concentrations [25]. More recently, the use of freeze-dried cells [2,7] or immobilized cells (this paper) have avoided many of the difficulties with suspended cells such as constant and consistent cell sample preparations.

Apart from the advantages of an immobilized-cell biosensor compared to suspension-culture biosensing, the method of latex immobilization has several additional advantages compared to other immobilization procedures. The immobilized biosensor consists of a multi-layer patch of immobilized cells and copolymer coated onto a polyester substrate (Figure 2) [23]. The latex forms a non-toxic microporous matrix that effectively traps the cells but does not adhere to them [42]. Once dried and cured, these patches form a strong yet flexible matrix. Cells immobilized in this manner maintain 80% of the original culturability, and culturability over 15 days is similar to or higher than that of suspended cells [23]. Our previous studies indicated that there was generally very low cell release from these patches, and that latex was non-toxic to *E. coli* [23]. Cryogenic scanning electron microscopy revealed that the cells and the latex form a micro-porous network in which the cells are physically trapped but not bound to the polymer phase [16,42]. Also, immobilization of cells in latex allows creation of very thin films, reducing the diffusion distance between the medium and the cells and permitting a relatively fast response of the immobilized patches to Hg(II). In contrast to other immobilized cell biosensors [22,27,33],

Table 1 Comparison of Hg(II) sensitivity and sample size for biological and chemical detection methods

Detection methods	Detection limit (nM)	Sample size (ml)	Reference
Biological methods			
Latex immobilized cells (bacterial luciferase)[a]	5×10^{-2}	10.0	This study
Suspended cells (bacterial luciferase)[a]	5×10^{-2}	2.0	[31,32,34,35]
Suspended cells (firefly luciferase)[b]	1×10^{-6}	0.1	[44]
ELISA[c]	25	0.1	[46]
Chemical or physical methods			
CVAFS[d]	1×10^{-4}	100	[3,4]
AES[e]	1×10^{-3}	50	[17,18]
RNAA[f]	1.5×10^{-2}	3	[18,19]

[a]*Mer-lux* plasmids in *E. coli*.
[b]*Mer-lucFF* plasmids in *E. coli*.
[c]Enzyme-linked immunosorbent assay.
[d]CVAFS, cold vapor atomic fluorescence spectroscopy.
[e]AES, atomic emission spectroscopy.
[f]RNAA, radiochemical neutron activation.

the mercury biosensor is easy to handle, cells remain viable and luminous for more than 78 h, and it was developed solely for single use. This eliminates the problems associated with immobilized-cell stability, cell outgrowth, and slow biosensor response times [29,39].

The maximum induced luciferase activity from a *mer-lux* fusion construct has been used as a measure of the Hg(II) concentration present in the cells [31,34,35]. Consistent with these findings, the maximum luciferase activity detected in the latex-immobilized cells increased as a function of mercury concentration (Figure 6). Therefore, to quantify the amount of mercury in an environmental sample, latex-immobilized cells would also be incubated with a set of mercury solutions of known concentration to serve as the standard for comparison to luciferase levels induced by the environmental sample. Because of the sigmoidal nature of the response, samples containing higher concentrations of mercury should be tested at different dilutions to avoid saturation of the detection system. Increasing the sensitivity of the assay to detect lower mercury levels would require increased luciferase induction at lower Hg(II) concentrations. LB medium supported higher induction levels of luciferase than the pyruvate buffer at the lower $HgCl_2$ concentrations, but LB medium contains about 0.1 nM total mercury contamination (unpublished data), which defines the lower detection limit of the assay. Assay buffers that support high luciferase induction at lower Hg(II) concentrations and are relatively mercury-free are needed to increase the Hg(II) sensitivity of the biosensor.

The addition of d-cysteine to immobilized cells increased the induced luciferase activity by approximately 100-fold. The mechanism by which cysteine increases Hg(II) induction of luciferase activity is unknown, but cysteine may prevent the binding of Hg^{2+} to cell surfaces [31], thereby decreasing mercury toxicity [10], and increase Hg^{2+} uptake into cells for more efficient interaction with the MerR regulatory protein. There was a remarkable difference in cysteine-enhanced induction of luciferase between suspended (3.5-fold) and immobilized cells (100-fold). The increased response of immobilized cells towards cystyl-Hg(II) for luciferase induction could be explained by the immobilization of the cells. In the suspension culture, cystyl-Hg(II) is evenly distributed among the cells, whereas in the immobilized biosensor, the mercury may have some binding affinity for the latex in addition to the cell surfaces, which is alleviated by formation of the cystyl-Hg(II) complex. Also, the uppermost layers of cells in the biosensor are potentially exposed to a greater number of cystyl-Hg(II) molecules per cell which may result in a higher level of induction.

The ability to store the immobilized cells beyond a few days after immobilization was an important factor in increasing the usefulness of the Hg(II) detection method. By freezing the patches immediately after immobilization in a solution of PBS and glycerol, the immobilized cells could be stored for at least 3 months before being thawed and used for Hg(II) detection. The induced luciferase activity observed after the freeze storage was very similar to the activity induced in newly immobilized cell patches. It should therefore be feasible to produce hundreds of immobilized cell patches at a time, freeze them for storage, and thaw prior to use for Hg(II) detection assays. In addition to freezing, patches were stored dry in a desiccator at room temperature for 14 days. This method would be analogous to using freeze-dried cell samples [2,7]. After rehydration of the patches, the levels of luciferase induction as a function of Hg(II) concentration decreased, and the lowest detectable Hg(II) concentration was 1 nM when compared to the 0.1 nM $HgCl_2$ sensitivity limit of freshly prepared patches. However, the range of Hg(II) concentrations that could be distinguished in the assay increased (1–10 000 nM) compared to freshly prepared patches (0.1–100 nM). The freshly prepared patches and dried patches could therefore have potentially different applications depending on whether the lowest sensitivity limit or the broadest range of Hg(II) detection was desired.

We predict that additional *E. coli* plasmids containing promoter-*lux* constructs for detecting other heavy metals and toxic molecules [5,6,9,12,26,40,43] could be used in the latex film-immobilized cell system that has been developed for mercury detection. In addition, constructs harbored by microorganisms other than *E. coli* may also be used as biosensors depending on the ability of the host to withstand the latex immobilization procedure and the efficiency of induced gene expression.

Acknowledgements

We thank O Selifonova and C Lange for helpful discussions and providing the strains used in this work. We also thank E Nater for the mercury analyses and valuable advice. This work was supported by National Science Foundation grant NSF/BES-9424063. OKL was partially supported by NIH training grant T32/GM08347.

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