Versatile biosensor vectors for detection and quantification of mercury

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Abstract

Three different whole cell biosensor constructs were made by fusing the mercury inducible promoter, P_{mer}, and its regulatory gene, merR, from transposon Tn21 with reporter genes luxCDABE, lacZYA, or gfp. In Escherichia coli these biosensor constructs responded to low levels of mercury by producing light, β-galactosidase or green fluorescent protein, respectively. Since the responses were quantitative, the constructs were used to quantify bioavailable mercury in different environments. The constructs were cloned into mini-Tn5 delivery vectors, thus enabling the transfer of the mer-lux, mer-lac or mer-gfp cassettes to a variety of Gram-negative bacteria. The mer-lux cassette was transferred to a Pseudomonas putida strain, which was used to quantify water-extractable mercury in contaminated soil. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Mercury; Whole cell biosensor; Soil contamination; lux; lacZ; gfp

1. Introduction

Many studies have used whole-cell biosensors to detect and measure the presence of metals in complex environments e.g. [1], many of which have focused on the detection of bioavailable mercury under both laboratory and environmental conditions [2–4]. Mercury, in the form of methyl mercury, is an environmental pollutant of great risk to public health. Measuring bioavailable mercury (Hg^{2+}) is essential for calculating methylation rates of mercury, and thereby predicting the bio-accumulation of methyl mercury in different environments [5].

All the mercury whole cell biosensors referred to above used a plasmid borne biosensor construct consisting of the mercury inducible promoter P_{mer} in combination with its regulatory gene merR, both obtained from the well described transposon Tn21 system [6]. However, some loss of sensitivity has been recorded when using plasmids instead of chromosomal inserts [7]. Furthermore, plasmids are sometimes lost from host organisms if selective pressure to maintain the plasmids is absent [8].

All mercury biosensor constructs so far use the host cell Escherichia coli. Paton et al. [9,10] found that different strains had remarkably different sensitivities to metals. It is therefore likely that some bacterial strains are better biosensor hosts than others. Furthermore it could be argued that E. coli is not the most ecologically relevant species to use as host, when examining the mercury content in environments like soil.

We therefore developed three flexible biosensor vectors for the detection and quantification of low concentrations of mercury. They provide the choice of the three different reporter gene systems, luxCDABE, lacZYA and gfp, combined with P_{mer}. Furthermore, the constructs were placed in mini-Tn5 delivery vectors, thus providing a choice of Gram-negative bacteria to be used as biosensor host cells.

2. Materials, methods and results

2.1. Strains, plasmids and culture conditions

All strains and plasmids are shown in Table 1. The E. coli strain MT102 was used as host strain in all DNA manipulation steps except for the final biosensor constructs using the mini-Tn5 plasmids: pUT-mer-lux, -mer-lac, -mer-gfp. These plasmids depend on the π protein for replication [18], and were therefore transformed into strain E. coli MT102-PIR [12]. The MT102-PIR strains hosting the different biosensor plasmids were then in turn used as...
donors in tri-parental filter matings (using NF1815(RK600) as a mobilizing strain) to insert the biosensor cassettes into the chromosome of different Gram-negative bacteria.

DNA, plasmids and fragments were analyzed, digested, manipulated, prepared and transformed by standard procedures [19] except in filter matings. Strains of *E. coli* were grown in LB broth [20] at 37°C, unless otherwise stated. Other strains used were grown at 30°C. The *pfu* polymerase was used in all PCR reactions (purchased from Stratagene, La Jolla, CA, USA). Stock solutions of HgCl$_2$ were aqueous solutions of Hg$_2^{2+}$.

All glassware in which the *mer* biosensor bacteria were used was washed, first in 0.1 M HNO$_3$, then rinsed in distilled H$_2$O, to avoid interference with induction of the biosensor construct by residual mercury.

Tri-parental filter matings were carried out to transfer the biosensor constructs to different Gram-negative bacteria (data not shown). This was done as described earlier [12], and biosensor transconjugants were selected on basal salts medium [21] containing 0.2% glucose and 50 µg ml$^{-1}$ kanamycin.

### 2.2. Cloning of the mer-lux gene cassette

The entire *mer-lux* gene cassette, containing *merR*, *P*$_{mer}$ and part of *merT* in an operon fusion with the lux*CDABE* genes, was excised from plasmid pRB28 using *EcoRI*–*PstI* double digestion and cloned into pLOW2, yielding the plasmid pLOW2-*mer-lux*. This step was carried out in order to generate NotI ends on the fragment, which were used in the next cloning step. The biosensor cassette was then excised from pLOW2-*mer-lux* as a NotI fragment and inserted into the unique NotI site of the mini-Tn5 delivery vector pUT-kn-res to generate pUT-*mer-lux* (Fig. 1).

#### 2.3. Cloning of the mer-lac gene cassette

$P_{mer}$ was excised from pRB28 using *EcoRI* and *BamHI* and inserted into pRS415 (a pBR322-based plasmid) thereby creating an operon fusion between $P_{mer}$ and the lac*ZYA* genes. This plasmid was called pRSA*mer-lac*. The mer-lac cassette was transferred from pRSA*mer-lac* into pLOW2 as a *PstI-SalI* fragment in order to generate NotI ends on the biosensor cassette. The cassette was then transferred into the mini-Tn5 delivery vector pUT-Kn-res as a NotI fragment to generate pUT-*mer-lac* (Fig. 1).

#### 2.4. Cloning of the mer-gfp gene cassette

Due to the lack of suitable cloning sites in the GFP vector pAG408, a different cloning strategy was used to introduce restriction sites on the *mer* repressor/promoter fragment. This was done by PCR.

The *mer* repressor/promoter was amplified from pRB28 using the following primers, *P*$_{mer}$*NotI*: 5'-GGCGGCGGCGC CGCGAATTCTGACGCTGCC-3’ and *P*$_{mer}$*Clal*: 5’-GGGCCATCGATGGATCCCCACTAGC-3’, to yield a 715-bp fragment encoding the *merR*, *P*$_{mer}$ and 29 codons of *merT*. These primers generate *NotI* and *Clal* sites (underlined) at the ends of the *mer* repressor/promoter. After a double digest using *NotI* and *Clal*, the resulting *NotI–Clal* fragment was ligated into plasmid pAG408 to yield a plasmid containing the mercury-inducible $P_{mer}$ in front of the *gfp* gene. This construct was called pAG-*mer-gfp*. A sec-

### Table 1

Plasmids and strains

<table>
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<th>Plasmid/strain</th>
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<th>characteristics</th>
<th>Reference</th>
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<td>pAG-g3-<em>mer-gfp</em></td>
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<td>Km$^R$, P$_{mer}$-gfp</td>
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<tr>
<td>pLOW2</td>
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<td>Km$^R$</td>
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<td>Ap$^R$, P$_{mer}$-lac*ZYA</td>
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<td>glu$^+$</td>
<td>[16]</td>
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<td>KT2440::P$_{mer}$-lux*CDABE, Km$^R$</td>
<td>This study</td>
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<td>MT102-PIR</td>
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<td>[12]</td>
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<td>NF1815</td>
<td><em>E. coli</em></td>
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Ap$^R$: ampicillin resistance, Cm$^R$: chloramphenicol resistance, Km$^R$: kanamycin resistance, Sm$^R$: streptomycin resistance.
ond PCR was then performed using primer PmerNoI as forward primer and a new primer, gfp-revNoI: 5'-ATGGGCGGACCATTCTATTGTT-3' (also containing a NoI site) as reverse primer. The resulting NoI fragment (containing merR, Pmer and gfp) was then cloned into pUT-Kn-res to yield pUT-mer-gfp (Fig. 1).

2.5. Induction assays

E. coli MT102-PIR(pUT-mer-lux) was grown overnight (ON) at 37°C in ABB1 media [22] containing 0.4% glycerol. Then, 1 ml of this culture was diluted into 40 ml of ABB1 media containing leucine and proline (no carbon source added). Three milliliter of this dilution was added to disposable plastic tubes (for luminometer counting). Mercury was added to obtain various total concentrations as shown in Fig. 2A. The tubes were mixed by gentle inversion and incubated at room temperature without shaking. Relative light units (RLU) per 30 s were measured at 80 min after incubation in a BG-P portable luminometer (MGM instruments, Hamden, USA), and plotted against mercury concentration (Fig. 2A).

An ON culture of E. coli MT102-PIR(pUT-mer-lac) grown as above was diluted 100-fold into fresh ABB1 medium containing 0.2% glucose and various concentrations of mercury. The bacteria were incubated for 4 h at 37°C with shaking. 0.5 ml was transferred to 1.5-ml Eppendorf tubes and placed on ice. Ten microliter of toluene was added to each tube and the sample was vortexed for 10 s. The toluene was evaporated at 37°C for 30 min. A β-galactosidase assay was then performed according to Miller [20]. β-Galactosidase levels were plotted against the concentration of mercury (Fig. 2B).

Induction of E. coli MT102-PIR(pUT-mer-gfp) was carried out in a diluted and modified LB-type medium containing the following compounds: tryptone 1 g l⁻¹, yeast extract 0.5 g l⁻¹ and NaCl 4 g l⁻¹ (LB4.10). An ON culture of E. coli MT102-PIR(pUT-mer-gfp) (grown in LB4.10 medium) at 30°C, was diluted 100-fold into fresh LB4.10 medium containing various concentrations of mercury and grown for 16 h at 30°C. A 3-ml sample from each Hg²⁺ concentration was washed twice and resuspended in 0.9% NaCl in order to minimize the background.
fluorescence of the LB media. The sample was then transferred to a luminescence spectrometer LS 50B (Perkin Elmer, Buckinghamshire, UK) and fluorescence was measured. The excitation wavelength was 395 nm and emission was measured at 509 nm. For each sample the optical density at 600 nm (OD 600) was determined to ensure compatible cell numbers. Relative fluorescent units (RFU)/OD 600 were plotted against the concentration of mercury (Fig. 2C).

2.6. Quantification of water-extractable mercury in soil

Pseudomonas putida KT2440::mer-lux was constructed by a tri-parental filter mating between P. putida KT2440, E. coli MT102-PIR(pUT-mer-lux) and helper strain NF1815(RK600). These biosensor cells were used to determine the water-extractable concentration of mercury in extracts of artificially contaminated soil. KT2440::mer-lux was inoculated into LB4.10 media and grown for 8 h at 30°C by which time cells had reached stationary phase. The cells were then diluted in ABB1 media (no carbon source added) to 6.4×10^7 cells ml^(-1). Aliquots of 2.97 ml were then distributed into disposable plastic tubes for luminometer counting.

The contaminated soil was made by adding 150 ml of double distilled H_2O, containing 0, 2.5, 10 or 25 μg of Hg^{2+}, to 8-ml centrifuge tubes containing 1 g of sieved and dried agricultural soil [3]. After 8 h, the soil was mixed with 7 ml of H_2O and Hg^{2+} was extracted for 1 h on a shaker at 25°C. The samples were centrifuged (15000 x g for 10 min). A series of 10-fold dilutions were made to ensure mercury concentrations in the extracts within the linear range of the standard curve. 30 μl of the soil extract dilutions was added to the biosensor cells. After 48 min, luminometer counts for cells with soil extracts were compared to luminometer counts from standard solutions (Fig. 3).

3. Discussion

Three biosensor cassettes were each situated in a mini-Tn5 delivery vector. The biosensor constructs all responded in a quantitative manner to different concentrations of mercury.

Adding to the versatility of these whole cell biosensors was the fact that they provide a choice of reporter genes. It appears that induction of the mer-lux and the mer-lac constructs were much more sensitive than induction of the mer-gfp construct (Fig. 1). However, the assay growth conditions were very different from one construct to another. Both the mer-lux and the mer-lac construct were grown in ABB1 media, compared to the modified LB media used for induction of E. coli MT102-PIR(pUT-mer-gfp), and the cell densities in the mer-gfp assay were much higher, compared to both mer-lux and mer-lac.

Others studies have shown that both complex medium and high cell densities decrease the amount of bioavailable mercury in such assays [4,23]. The differences in assay conditions reflect the differences between the three reporter genes. Detection of GFP required long incubation (16 h optimal) and detection of light gave fluctuating results, whereas cells containing the mer-lux construct were growing. Therefore the assay conditions were changed depending on which reporter gene was used. This complicates any comparison of sensitivity between the different biosensor constructs. However, the lux and lacZ are known as sensitive reporter genes in whole cell biosensors [24], whereas the gfp gene is known for its applications in in situ microbial ecology [25,26].

The mer biosensor constructs described in this study can be conjugated into a variety of Gram-negative bacteria, where integration into the host chromosome will ensure a stable construct, a constant copy number and maintenance even under non-selective conditions. All three mini-Tn5 constructs were successfully transferred into different Gram-negative bacteria such as Enterobacter aerogenes, Enterobacter chloacae, two strains of E. coli, Pseudomonas fluorescens and P. putida (data not shown).

Soil bacteria like P. putida can readily be used to host biosensor constructs that are sensitive to mercury (Fig. 3). The results showed that only a small fraction of the mercury (less than 3%) was extracted from the soil. The water-extractable amounts mercury found in soil in this study, using KT2440::mer-lux, are consistent with findings by Rasmussen et al. [3]. They found that approximately 50 ng of mercury was water-extractable from this soil spiked with 2.5 μg g^(-1) soil. In this study that number was 67 ng (Fig. 3). In the study by Rasmussen et al., E. coli hosted the plasmid-borne mer-lux construct. We are currently investigating the sensitivity and robustness of P. putida and several other potential host strains.

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Fig. 3. Measurement of the water-extractable Hg^{2+} vs. the total Hg^{2+} concentration in artificially contaminated agricultural soil. The extractable Hg^{2+} was measured by the newly constructed biosensor strain P. putida KT2440::mer-lux. <5: below detection limit (5 ng (g soil)^{-1}).
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References