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# Induction of bacterial mercury- and copper-responsive promoters: functional differences between inducible systems and implications for their use in gene-fusions for in vivo metal biosensors

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## SUMMARY

We have compared the induction by the cognate metal salts of two promoters responsible for metal-resistance gene expression in bacteria. The mercuric ion resistance promoter, $P_{merTPAD}$, of transposon Tn*501* and the copper resistance promoter, $P_{pcoE}$, from plasmid pRJ1004 were separately cloned to express the *lacZ* gene under the regulation of their normal *trans*-acting elements. The *lux* genes of *Vibrio fischeri* were also expressed from $P_{merTPAD}$. The induction of $P_{merTPAD}$ gave a hypersensitive profile, as reported previously: the apparent Hill coefficient was 2.6 when using β-galactosidase activity as a measure of *lacZ* gene expression. In contrast, the induction of $P_{pcoE}$ was hyposensitive, with an apparent Hill coefficient of 0.63 for induction of β-galactosidase activity, and this may be related to the role of copper as an essential micronutrient. These response profiles suggest that transcriptional fusions of the *merTPAD* promoter allow the construction of strains that are suitable for detecting threshold levels of mercuric ions, but not for accurate determinations of mercuric ion concentrations across a wide range. In contrast, transcriptional fusions to the *pcoE* promoter are well suited to determination of the concentrations of copper salts. The comparison of induction profiles of $P_{merTPAD}$ using *lacZ* or *lux* reporter genes, show different stimulus-response curves, probably due to differing instrument sensitivities. These results have practical implications in the construction of whole cell gene-fusion biosensors for the detection and quantitation of heavy metals.

## INTRODUCTION

The use of transcriptional fusions between a reporter gene and a regulated promoter have been developed and widely used to study the mechanisms of regulation of gene expression [17]. Historically such fusions used *lacZ, galK* or other genes and required subsequent assay of the gene product. More recently, the *lux* genes of luminescent bacteria have been used, as these can be assayed in vivo in real time by detecting photon emission, and have potential for use in environmental or industrial situations—as illustrated in references listed in [6]. Gene fusion technology is proposed for use in biosensors for the detection of environmental pollutants and of industrial waste products, and the *lux* genes are particularly promising for this application because of the ease of assay of their expression. However, the response of a reporter gene to different concentrations of inducer has not been studied in detail for many systems of environmental or industrial relevance [6].

We were interested in establishing the potential for using the *lux* genes to assay two regulated systems. The first was the well-characterized mercuric ion resistance promoter of transposon Tn*501* [7]; the second was one of two recently-identified promoters from the *pco* copper resistance determinant from *E. coli* (D.A.R. and N.L.B., unpublished). In each case the reporter system might be used to determine concentrations of a heavy metal in environmental or industrial samples. Several inducible metal resistance determinants have been identified in bacteria, and their expression is often regulated by one or a few heavy metals [18]. Other workers have recently made gene fusions with the regulatory regions of metal resistance determinants [2,3,16]. In this paper we describe the assay of the $P_{merTPAD}$-*lacZ*, $P_{merTPAD}$-*lux* and $P_{pcoE}$-*lacZ* fusions against increasing concentrations of the cognate heavy metal ion and correlate these with growth rate of the cells. The data indicate that the copper-resistance system has a gradual response to increasing copper ion concentration, whereas the mercury resistance system has a sharp threshold response. The biological reasons for this difference may be due to the requirement for copper as a micronutrient, in contrast to mercury which apparently has no benefit to the bacterial cell. The potential application of these systems for the construction of biosensors will also differ.

## MATERIALS AND METHODS

### *Bacterial strains and plasmids*

All work in this paper utilized *E. coli* TG2 (K12, Δ*lac-pro, supE, thi, hsdD5, recA*1, Tn*10* [F′, *traD36, proA*$^+$*B*$^+$, *lacI*$^q$,

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*lacZ* ΔM15]) (T. Gibson, personal communication). The plasmids used are given in Table 1.

*Construction of reporter plasmids*

The $P_{merTPAD}$-*lacZ* reporter plasmid, pRZH18, has been described previously [7]. The $P_{merTPAD}$-*lux* reporter fusion, pUCD615-$P_{merTPAD}$, was constructed by purifying the *Eco*RI-*Lsp*I fragment of Tn*501* containing the *merR* gene and $P_{merTPAD}$, incubating the fragment with Klenow DNA polymerase and all four deoxyribonucleoside triphosphates, and cloning the fragment into the *Sma*I site of plasmid pUCD615 [12]. The orientation of the insert was determined by restriction endonuclease digestion. A partial *Sau*3AI digest of pPA256 [13] was cloned in the *Bam*HI site of plasmid pMU575 [1] and blue colonies were selected on agar containing 4 mM $CuSO_4$. An 800-bp fragment was present in the resulting plasmid, the identity and orientation of which indicates that the $P_{pcoE}$ promoter of the copper-resistant determinant *pco* [13] expressed the *lacZ* gene. As the exact location of the promoter was not known at the time of these experiments, a corresponding $P_{pcoE}$-*lux* construct was not made.

The plasmid pPA87 (*pco* in a double pBR322 replicon and conferring copper resistance [14]) was co-introduced with $P_{pcoE}$-*lacZ* reporter plasmid into *E. coli* TG2 in order to provide plasmid-borne regulatory functions and to allow measurements to be made at high copper concentrations.

*DNA manipulations*

Klenow DNA polymerase and some restriction endonucleases were obtained from Boehringer Corporation (London) Ltd; DNA ligase and *Lsp*I were obtained from Northumbrian Biologicals, UK; other restriction endonucleases were obtained from New England Biolabs Inc., UK or Gibco-BRL, UK. All enzymes were used in accordance with the manufacturers' instructions. Purification of plasmid DNA as vectors for cloning, purification of DNA fragments by gel electrophoresis, ligation reactions, and transformation of bacteria were according to procedures described elsewhere [10,15].

*β-galactosidase assays*

Overnight shake cultures of *E. coli* TG2 each containing the appropriate plasmid constructs were grown at 37 °C in either (i) minimal medium M9, supplemented with casamino acids and glucose, for $P_{merTPAD}$ constructs or (ii) Luria–Bertani (LB) medium, treated with Chelex resin (Biorad) according to the manufacturers' instructions, for $P_{pcoE}$ constructs. In each case growth was with selection for the plasmid antibiotic markers. Minimal medium is preferable for metal ion induction experiments, but will not support the high concentrations of uncomplexed Cu(II) salts required to induce the $P_{pcoE}$ promoter fully. The overnight culture was diluted into 50 volumes of prewarmed medium, and grown until $A_{600}$ was 0.6–0.7. The appropriate concentration of metal salt was then added to those cultures which were to be induced and growth was continued for 1 h. The cultures were quickly chilled to 0 °C. In assays of induction of the copper-responsive promoter, cells were rapidly harvested by centrifugation, resuspended in the original volume of LB medium, but lacking cupric ions, and assayed immediately. This minimizes interference with the assay by

TABLE 1

Plasmids used in this study

| Plasmid | Genotype/ phenotype[a] | Size (kb) | Remarks | Source/ reference |
|---|---|---|---|---|
| pMU575 | $Tp^r$ (*lacZ*) | 15 | IncW plasmid with promoterless *lacZ* gene | [1] |
| pUCD615 | $Km^r$ $Ap^r$ (*lux*) | 17.5 | Sa-derived plasmid with promoterless *lux* operon | [12] |
| pPA87 | $Cu^r$ $Ap^r$ $Tc^r$ | 21.1 | 12.5-kb *Hin*dIII fragment of pPA110 in a tandem repeat of pBR322 | [14] |
| pPA256 | $Ap^r$ | 8.2 | 3.9-kb *Eco*RV fragment of pPA87 cloned into pBR322 | [14] |
| pRJ1004 | $Cu^r$ | 120 | Natural copper resistance plasmid, origin of *pco* determinant | [19] |
| pRZH18 | $Km^r$ *lacZ* | 15.7 | pRZ5255 containing *merR* and $P_{merTPAD}$ of Tn*501* | [7] |
| pMU-$P_{pcoE}$ | $Tp^r$ lacZ | 15.8 | pMU575 containing the *pcoE* promoter of *pco* | This study |
| pUCD615-$P_{merTPAD}$ | $Km^r$ $Ap^r$ *lux* | 18.2 | pUCD615 containing *merR* and $P_{merTPAD}$ of Tn*501* | This study |

[a] Ap, ampicillin; Km, kanamycin; Tc, tetracycline; Tp, trimethoprim.

copper salts. The activity of β-galactosidase was measured in permeabilized cells as described by Miller [8].

*Luminescence measurements*

Cultures for the luminescence assay were grown as were those for the β-galactosidase assay, with the exception that cells were not washed to remove metal ions immediately prior to the assay. The luminescence of cell cultures was measured in a Lumac luminometer model M1500, kindly loaned by the manufacturers, Lumac Ltd, UK.

*Bacterial growth rates*

These were measured at several different concentrations of metal ion as changes in $A_{600}$ with time in mid-exponential growth.

## RESULTS AND DISCUSSION

*Induction of β-galactosidase activity from $P_{merTPAD}$-lacZ with increasing Hg(II) concentration*

The β-galactosidase activity expressed from pRZH18 in response to increasing $HgCl_2$ concentration is shown in Fig. 1(A). These cells contained no structural genes for mercury resistance, and showed normal mercury sensitivity. The induction from 10% to 90% of fully-induced levels occurred across a narrow range of concentration (c. 4-fold) and the data fitted to a line with an estimated Hill coefficient [5] of 2.6. Fig. 1(A) shows the growth rate of the cells at the different mercuric ion concentrations and it was observed that $P_{merTPAD}$ is almost fully induced at Hg(II) concentrations below those causing a reduction in growth rate.

The biological implications of this are that the mercury resistance genes would be fully-induced from $P_{merTPAD}$ at sub-toxic concentrations of mercuric ions. The expected curve of the same mid-point value but showing normal Michaelis–Menten kinetics (i.e. a Hill coefficient of 1.0 [5]) is also shown in Fig. 1(A) and indicates that a response curve with a Hill coefficient of 1.0 and with the same mid-point sensitivity of induction, would not give full induction of the resistance genes before the growth rate was substantially reduced. A hypersensitive response of $P_{merTPAD}$ has previously been observed in vitro [11] and in vivo [2]. This is the first time that the induction profile has been related directly to the growth of cells in the same medium.

*Induction of β-galactosidase activity from $P_{pcoE}$-lacZ with increasing Cu(II) concentration*

There is increasing expression of β-galactosidase activity with cupric ion concentration (Fig. 1(B)). The response in this case was gradual with increasing metal ion concentration. These cells contain a functional metal resistance determinant (*pco*) on the plasmid pPA87 supplying the regulatory functions *in trans*. The shape of the stimulus-response curve was not due to the presence of the *pco* determinant, as was demonstrated by supplying the regulatory functions on pPA256 (which does not confer resistance). In this case the response curve was the same, but extended only up to 3 mM $CuSO_4$, beyond which the cells did not grow (data not shown).

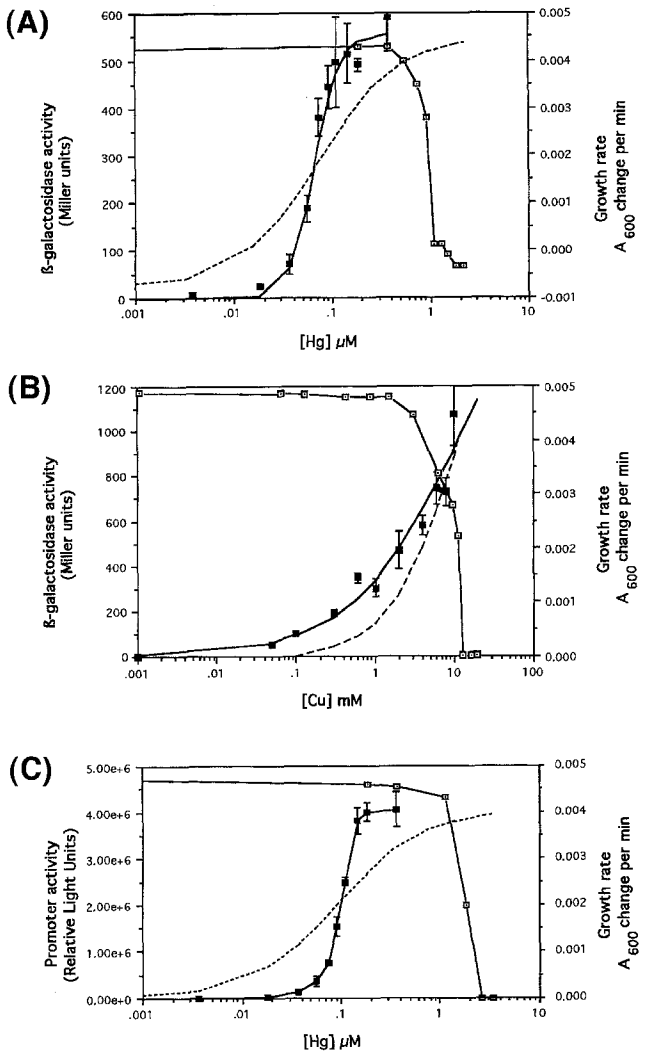


Fig. 1. Expression of reporter genes with increasing metal ion concentration. Each measurement (solid boxes) was done in triplicate and bars indicate the standard error. Stimulus-response curves were fitted by the method of Hill [5]. Dotted lines show the expected stimulus-response curve of the same mid-point that would obey Michaelis–Menten kinetics (Hill coefficient = 1.0). The growth rate of the bacterial strain against metal ion concentration is indicated by open boxes. (A) Expression of the *lacZ* gene from $P_{merTPAD}$ with increasing $HgCl_2$ concentration in strain *E. coli* TG2 (pRZH18). (B) Expression of the *lacZ* gene from $P_{pcoE}$ with increasing $CuSO_4$ concentration in strain *E. coli* TG2 (pMU-$P_{pcoE}$). (C) Expression of the *lux* genes from $P_{mer}$ with increasing $HgCl_2$ concentration in strain *E. coli* TG2 (pUCD615-$P_{merTPAD}$).

The data points did not lie on a sigmoidal response curve, but on a hyperbolic curve. This indicates that the maximum response observed in these experiments, at which a significant reduction in growth rate occurs, is not the maximum possible response of the promoter. At 10 mM $CuSO_4$ concentration, the slope of the induction curve is close to its maximum, suggesting to us that the data fit to the bottom half of a sigmoidal response. With this assumption, the best fit of the data is to a curve with an apparent Hill coefficient of 0.63. The expected curve with the same mid-point and obeying Michaelis–Menten kinetics is shown in Fig. 1(B).

From these data, it appears that the copper-response system has evolved to utilize only the lower part of the sigmoidal response curve, where the rate of increase of response with increasing Cu(II) concentration is low. The biological effect of this would be to give a fine adjustment of expression of the copper resistance genes across a wide range of external Cu(II) concentration.

*Functional implications of the different stimulus-response curves for mercury and copper resistance genes*

The different responses of the promoters from the *mer* and *pco* resistance determinants can be related to the biological properties of the metals. Microorganisms are often exposed to gradients of compounds in the environment, whether these are nutrients, antibiotics or toxic metals. Mercury in its ionic form is highly toxic, and there is no known advantageous biological role for Hg(II). Thus, it is advantageous for a mercury resistance determinant to eliminate Hg(II) from the cell as efficiently as possible even at subtoxic metal concentrations. The hypersensitive response of $P_{merTPAD}$ permits this by giving full induction of the resistance genes across a narrow range of Hg(II) concentration (10–90% induction occurs across a 4-fold change in Hg(II) concentration). The advantages of using a hypersensitive system are (i) that full induction at subtoxic metal concentration can be obtained without induction occurring unnecessarily at very low Hg(II) concentration, and (ii) that low Hg(II) concentration may precede high Hg(II) concentrations and the system is already fully-induced to allow for a subsequent increase. Thus, induction of $P_{merTPAD}$ initiates at about 0.02 $\mu$M $HgCl_2$ and maximum induction occurs by 0.2 $\mu$M, whereas a 10% decrease in growth rate does not occur until 0.7 $\mu$M. The mercury resistance genes are therefore only induced when they are needed to assure the organism's survival.

In contrast, Cu(II) is an essential micronutrient, and intracellular concentration of copper ions, as Cu(II) or Cu(I), need to be maintained. Full and rapid induction of the copper export system encoded by the resistance genes is not possible, since it could deplete the cell of essential copper. A more gradual response allows the export of copper ions at rates which may allow intracellular copper concentrations to be maintained within optimal limits. Our data indicate that the *pco* system has evolved to allow an even more gradual response that normally obtained in biological systems obeying Michaelis–Menten kinetics. This effect is observed in two ways. First, only the bottom part of the sigmoidal stimulus-response curve is used, where the slope is lower than at the midpoint; and, second, the system is hyposensitive (i.e. has a low apparent Hill constant), thus extending the range of response to very low copper concentrations for the same mid-point response.

*Comparison of responses of the* $P_{merTPAD}$-lacZ *and* $P_{merTPAD}$-lux *fusions to induction by mercuric ions*

The stimulus-response curve for expression of the *lux* genes from the promoter $P_{merTPAD}$ are shown in Fig. 1(C). The mid-point of the response is at 0.1 $\mu$M $HgCl_2$, corresponding closely with that of the response mid-point for the *lacZ* gene in Fig. 1(A). However, the curve is much steeper when Relative Light Units (RLU) are measured using a luminometer, (10–90% induction occurs across a 2-fold range of Hg(II) concentration) giving a best-fit curve [5] with an apparent Hill coefficient of 5.1. This value differs from the 4- to 5-fold range obtained with *lacZ* (above), by measurement of transcript production in vivo [11], and by other groups using $P_{mer}$-*lux* fusions [2]. This difference in measurement of induction may be due to underestimates of RLU at low induction coupled with saturation of the RLU signal at higher induction levels; therefore, we do not ascribe any biological significance to this. Other workers [2] using different instrumentation have measured 10–90% induction occurring across a 4.2-fold range of mercuric ion concentration. Both *lux* and *lacZ* reporter genes are inhibited by high concentrations of Hg(II) [2,9]. Differences between the response of the *lux* operon and other reporter genes to induction by metal-responsive elements have also been reported by others [3].

*Gene fusions as environmental biosensors*

The data presented in this paper show that the mercury- and copper-inducible promoters from bacterial resistance determinants respond in different ways to external concentrations of the inducing metal ion. Fusions of such promoters with *lux* genes are proposed as useful in determining environmental concentrations of heavy metals [3,4,16]. Our data suggest that *mer-lux* fusions will be more useful in determining threshold concentrations of Hg(II) ions required to induce the promoter, whereas *pco-lux* fusions may be useful for determining actual concentrations of Cu(II). In all such uses, only biologically-available metal concentrations are measured, and the effects of other contaminants in environmental samples may affect the quality of the data obtained.

The possibility exists to manipulate the stimulus-response curves of the promoters by genetically modifying the promoters or the *trans*-acting proteins in order to alter the response. Such modifications could in principle include the replacement of the hypersensitive response of $P_{merTPAD}$ by a regular Michaelis–Menten response. It is possible that such altered responses could be selected in vivo by coupling the promoters to suitable selectable genes.

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