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SHORT CONTRIBUTION

K. B. Riether · M.-A. Dollard · P. Billard

Assessment of heavy metal bioavailability using *Escherichia coli zntAp::lux* and *copAp::lux*-based biosensors

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Abstract To determine the amount of metals detectable by bacteria, two plasmids were constructed in which the metal-inducible *zntA* and *copA* promoters from *Escherichia coli* were fused to a promoterless *Vibrio fischeri luxCDABE* operon. The luminescence response of *E. coli* bearing these constructs was studied as a function of the concentration of several heavy metals and was shown to be influenced by cell growth phase. The *zntAp::lux* fusion is induced mainly by salts of cadmium, lead, mercury and zinc, with significant induction by other metal ions, whereas the specificity of *copA* induction is restricted to copper and silver. In optimized assay conditions, metals could be detected at threshold concentrations ranging from nanomolar to micromolar, with maximal induction observed after only 60–100 min incubation. The ability of these biosensor strains to distinguish bioavailable quantities of metals in a sample makes them good candidates as useful tools to monitor metal contamination in environmental samples.

Introduction

The evaluation of heavy metal contamination in different environmental compartments traditionally relies on sensitive physical and chemical techniques such as atomic absorption spectroscopy or mass spectrometry. However, there are limits to their use due to the lack of information on the bioavailability and biological activity of the metal contaminant. In the past decade, the knowledge gleaned on the regulatory mechanisms of bacterial metal-regulated operons (Nies 1999; Bruins et al. 2000) has been exploited to develop rapid assays that allow the detection of the bioavailable (or bioactive), as opposed to total, metal fraction of contaminated samples. These assays use bioluminescent bacterial sensors, in which a luminescence-encoding reporter gene (the promoterless *lux* genes of luminescent bacteria such as *Vibrio fischeri* or the *luc* gene of *Photinus pyralis*) is inserted downstream of a metal-inducible gene, so that light is produced when the bacteria are exposed to the cognate metal ions (for a review, see Köhler et al. 2000).

In *Escherichia coli*, resistance to toxic lead and cadmium ions, and homeostasis of zinc, is mediated, at least in part, by the *zntA* gene, which encodes an integral membrane protein of the CPx-type ATPase family that has been shown to be responsible for the export of Zn(II) Cd(II) and Pb(II) ions from the cells (Beard et al. 1997; Rensing et al. 1997). A second P-type ATPase called CopA acts as an efflux pump of copper ions and contributes to the maintenance of safe levels of this essential trace element in bacterial cells (Rensing et al. 2000). The expression of the *zntA* and *copA* genes is controlled by ZntR and CueR, respectively, two metalloregulatory proteins belonging to the MerR family of transcription regulators (Outten et al. 1999, 2000).

In this study, we constructed *lux* transcriptional fusions to the *zntA* and *copA* genes to create heavy metal luminescent reporter strains, whose detection of bioavailable metal ions under different assay conditions are examined.

Materials and methods

Materials

The sources of the chemicals and related reagents used in this study are as follows: $CoCl_2$, $CuSO_4$, $Pb(NO_3)_2$, β-glycerophosphate and antibiotics were purchased from Sigma (St Louis, Mo.). $AgNO_3$, $CdCl_2$, $HgCl_2$, $K_2Cr_20_7$, K_2CrO_4, $KSbC_4H_4O_7$, $NiCl_2$, $ZnSO_4$ and EDTA were from Merck (Darmstadt, Germany). MES [2-(*N*-morpholino)ethane-sulfonic acid] and Na_2S were from Fluka. MOPS [3-(*N*-morpholino)propane-sulfonic acid], synthetic oligonucleotide primers and *Taq* polymerase (ExtrapolII) were from Eurobio (Les Ulis, France). DNA modifying enzymes were obtained from either New England Biolabs or MBI Fermentas. All metals were of analytical grade.

K.B. Riether · M.-A. Dollard · P. Billard (✉)
U.R. Ecotoxicité, Biodiversité, Santé Environnementale,
Université de Metz, Campus Bridoux – Rue du Général Delestraint,
57070 Metz Borny, France
e-mail: billard@bridoux.sciences.univ-metz.fr
Tel.: +33-3-87378513 , Fax: +33-3-87378512

Bacterial strains and media

E. coli DH10B [F- *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), (ϕ80d*lac*-ZΔM15), Δ*lac*X74, *end*A1, *rec*A1, *deo*R, *ara*D139, Δ(*ara-leu*) 7697, *gal*U, *gal*K, λ-, *nup*G, *rps*L] was used as the host strain for cloning experiments and for propagation of recombinant plasmids. Strain MG1655 (Jensen 1993) was the recipient for plasmids pZNT-lux and pCOP-lux and the resulting metal sensor strains were used in all bioluminescence assays. The strains were routinely grown at 37°C in Luria-Bertani (LB) broth (Difco), supplemented with ampicillin (50 µg/ml), or tetracycline (10 µg/ml) when required. Luminescence-based assays were performed using a modified glycerol-glycerophosphate (GGM) medium (Hughes and Poole 1991) (glucose 0.5%, 40 mM MOPS, 1 mM $MgCl_2$, 18 mM NH_4Cl, 13 mM KCl, 5 mM K_2SO_4, 0.07 mM $CaCl_2$, 5 mM disodium β-glycerophosphate) in which glucose was used as the carbon source instead of glycerol, and without added trace metals. Metal-supplemented media were prepared by adding the appropriate volumes of filter-sterilized metal stock solutions prepared in Milli-Q purified water (Millipore, Molsheim, France).

Construction of plasmids pZNT::lux and pCOP::lux

General procedures for strain and plasmid construction were performed according to Sambrook et al. (1989). The *zntA* promoter region was amplified from MG1655 genomic DNA by PCR using modified upstream (5′-CGGGATCCGCTCGCTGTATCTCTG-3′) and downstream (5′-GAGGGGAATTCTTGCCGTGATTGTC-3′) primers that created *Eco*RI and *Bam*H1 sites (underlined sequences) at positions –95 and +34, respectively, with respect to the start codon. The resulting PCR product was digested with *Bam*H1 and *Eco*RI and ligated into *Bam*H1-*Eco*RI-digested pUCD615 (Rogowski et al. 1987), to yield plasmid pZNT-lux, in which the complete *luxCDABE* operon from *V. fischeri* was controlled by the *zntA* promoter. The same procedure was followed to create plasmid pCOP-lux, a pUCD615-based vector containing a 467-bp PCR-amplified promoter/operator of *copA* by using the following primers: 5′-GTAAGCCGGATCCACTGCCTGC-3′ and 5′-CGGAATTCTGCACTGGCACCGTCCCGG-3′.

Preparation of cells and luminescence measurements

Standard induction assays were conducted as follows: overnight cultures (16 h) of *E. coli* MG1655(pZNT-lux) and MG1655 (pCOP-lux) were harvested by centrifugation in stationary phase, washed in LB and suspended in LB to obtain an optical density at 600 nm (OD_{600}) of 10. The bacterial suspension was then adjusted to a final OD_{600} of 0.2 by dilution into GGM (pH=6.5). For luminescence measurements, 20 µl of tenfold concentrated metal salt solutions were added to 180 µl of cell suspension in opaque white 96-well microtiter plates (Dynatech, Germany). Plates were incubated at 30°C in a Lumistar luminometer (BMG LabTechnologies, Germany) and bioluminescence was recorded every 20 min for at least 3 h without shaking. All experiments were conducted in duplicate, each sample being measured for 10 s. 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) after 80 min incubation.

Optimization of the assay conditions

Four series of experiments were conducted with MG1655(pZNT-lux) exposed to different concentrations of cadmium, lead, zinc and mercury in parallel, to optimize the assay conditions. Cell preparation and luminescence measurements were performed as for the standard induction assay, except for the parameter being tested. Influence of the growth phase was assessed using cells harvested at different culture times from a continuously growing LB culture, from exponential until stationary phases. The influence of medium composition was tested by replacing GGM minimal medium by LB medium. When testing the influence of cell density, the final OD_{600} in the assay was adjusted to values ranging from 0.05–10. The luminescence response was measured at different pHs ranging from 5 to 8.5 in GGM buffered with either MES or MOPS.

Effect of EDTA and Na_2S

The effect of two metal complexing agents, EDTA and Na_2S, on the luminescence response of MG1655(pZNT-lux) and MG1655 (pCOP-lux) was investigated both in the absence and presence of metals. Metal concentrations chosen for this purpose [10 µM $Pb(NO_3)_2$ and $CdCl_2$, 100 µM $ZnSO_4$, 150 µM $CuSO_4$] were high enough to ensure a toxic response and a nearly total inhibition of bacterial sensor luminescence. EDTA and Na_2S were diluted into the metal solution with chelate/metal molar ratios ranging from 0 to 2 and incubated for 2 h at room temperature to ensure metal complexation. Luminescence measurements were carried out as for the standard induction assay. Results are presented as the induction coefficients.

Results

Optimization of luminescence assay conditions

Parameters that could influence the biosensor luminescence response were optimized using MG1655(pZNT-lux) as the model. The growth phase at which bacteria were harvested dramatically affected the magnitude of the luminescence response. Maximal induction could be attained with early stationary-phase cells, which produced several fold higher luminescence than that of exponentially or late stationary phase cells (Fig. 1). However, for practical reasons, further experiments were conducted with overnight (16 h) cultures of the reporter strain, which still display significant metal induction. Medium composition, cell density in the assay, and pH also affected the induction of MG1655(pZNT-lux). The GGM minimal medium, which contains ß-glycerophosphate instead of inorganic phosphate, allowed a more sensitive metal detection than LB. A correlation was found between cell density and light emission for OD_{600} values ranging from 0.05–0.5, but higher cell concentrations in the assay reduced the observed luminescence level, possibly because of metal adsorption by cell wall components, or light absorption due to the turbidity of the bacterial suspension. In all cases, maximal induction occurred between 60 and 160 min after addition of the metal (data not shown). Standard assays were then performed with stationary-phase cells, suspended in GGM (pH=6.5) at a cell density corresponding to OD_{600}=0.2, and induction coefficients were calculated after 80 min incubation.

Induction of *zntAp::lux* and *copAp::lux* by heavy metals

The bacterial sensors were induced as described below in the presence of increasing concentrations of different

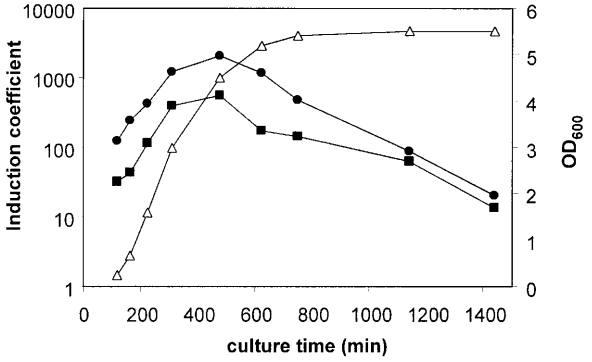

Fig. 1 Influence of the cell growth stage on the response of MG1655(ZNT-lux) to metals. Growth curve of the source culture (*open triangles*) and induction coefficients of MG1655(pZNT-lux) when exposed for 80 min to 0.3 µM $CdCl_2$ (*black squares*) and 1 µM $Pb(NO_3)_2$ (*black circles*) are shown

metals and metalloids. Among these, Cd(II), Pb(II), Zn(II), Hg(II), and to a lesser extent Co(II), Ni(II), SbO_2^-, CrO_4^{2-} and $Cr_2O_7^{2-}$, induced luminescence of the *zntAp::lux* fusion (Fig. 2). Metal concentrations that caused the highest luminescence induction were 0.3 µM $CdCl_2$, 1 µM $Pb(NO_3)_2$, and 30 µM $HgCl_2$ and $ZnSO_4$. When exposed to higher concentrations, light emission rapidly declined, suggesting a toxic metal level was reached in the assay. The lowest detection threshold was obtained with cadmium, which elicited significant (more than twofold) luminescence induction at 10 nM. Other metal ions were detected at higher concentrations in the following order: Pb(II)>Hg(II)>Zn(II)>Co(II)>Ni(II)= SbO_2^->CrO_4^{2-}=$Cr_2O_7^{2-}$ (Fig. 2b). In contrast to *zntA*, the induction specificity of the *copA* gene seems to be restricted to only two metals, Cu(II) and Ag(I) (Fig. 2c). The *copAp::lux* fusion was primarily sensitive to Ag(I), with a detection threshold of 0.1 µM, whereas a significant response to Cu(II) was triggered between 0.3 and 1 µM.

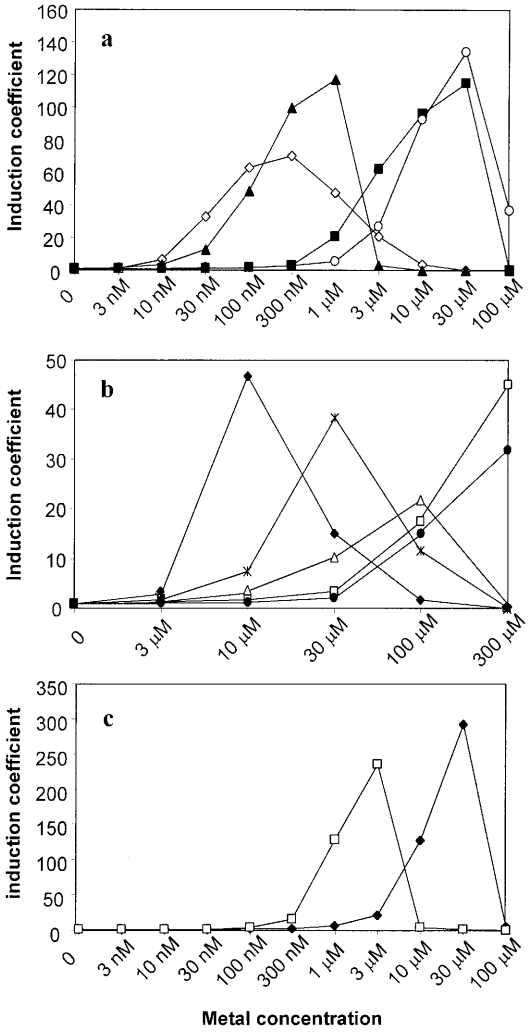

Fig. 2a–c Induction profiles of MG1655(pZNT-lux) and MG1655(pCOP-lux) in the presence of different metals. **a**, **b** The response of MG1655(pZNT-lux) incubated for 80 min with $CdCl_2$ (*open diamonds*), $Pb(NO_3)_2$ (*black triangles*), $ZnSO_4$ (*open circles*), $HgCl_2$ (*black squares*), $CoCl_2$ (*black diamonds*), $NiCl_2$ (*asterisks*), $KSbC_4H_4O_7$ (*open triangles*), K_2CrO_4 (*black circles*) and $K_2Cr_20_7$ (*open squares*). **c** Induction profiles of strain MG1655(pCOP-lux) after 80 min exposure to $AgNO_3$ (*open squares*) and $CuSO_4$ (*black diamonds*)

Bioavailability of metals

To assess the ability of the two bacterial sensors to distinguish available and immobilized forms of metals, the metal complexing agents EDTA and Na_2S were added to metal solutions before the induction assay. Experiments conducted with $CdCl_2$ and $Pb(NO_3)_2$ concentrations that cause maximal induction of *zntA* (0.3 µM and 1 µM, respectively) showed that the addition of EDTA resulted in a dramatic decrease in light emission (not shown). However, EDTA on its own had a strong negative effect on the basal light production of MG1655(pZNT-lux) (Fig. 3a), probably due to the chelation of essential trace elements in the medium used. To determine whether reduced light emission observed in the above experiment was due to metal chelation, similar assays were performed with high, toxic, metal concentrations. As expected, the addition of EDTA reduced the metal toxicity to the two biosensor strains, as judged by the progressive restoration of luminescence that occurred with EDTA/metal molar ratios ranging from 0–1 (Fig. 3b). Maximal light recovery was observed for equimolar ratios of EDTA and metals. Higher EDTA/metal ratios in the assay, however, resulted in a near total inhibition of light emission.

In contrast to EDTA, Na_2S was found to significantly reduce background luminescence of MG1655(pZNT-lux) only at concentrations exceeding 10 µM (Fig. 3a). The pattern of luminescence in response to added Na_2S is presented in Fig. 3c. The luminescence response was progressively recovered by increasing concentrations of Na_2S up to a Na_2S/metal molar ratio of 2, except for

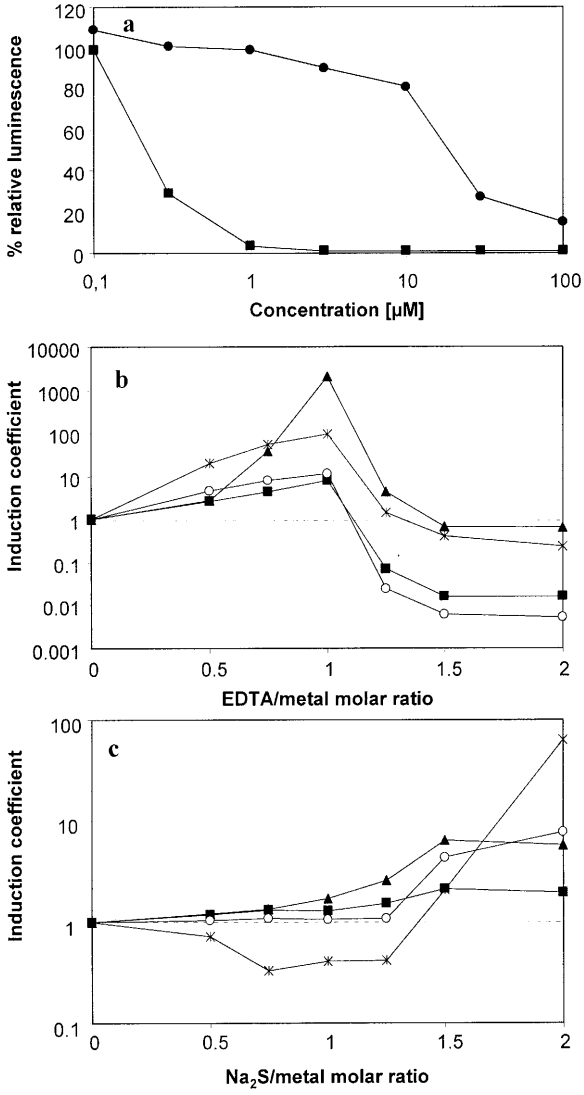

Fig. 3a–c Effect of two metal complexing agents on the response of MG1655(pZNT-lux) and MG1655(pCOP-lux). **a** Inhibition of background luminescence of strain MG1655(pZNT-lux) by Na_2S (*black circles*) and EDTA (*black squares*). Effect of increasing concentrations of **b** EDTA and **c** Na_2S on the luminescence of MG1655(pZNT-lux) exposed for 80 min to 10 µM $Pb(NO_3)_2$ (*black triangles*), 10 µM $CdCl_2$ (*black squares*), 100 µM $ZnSO_4$ (*open circles*) and MG1655(pCOP-lux) exposed to 150 µM $CuSO_4$ (*asterisks*)

strain MG1655(pCOP-lux) which displayed a biphasic response curve.

Discussion

In this study, two luminescent *E. coli* strains bearing plasmid-based *zntAp::lux* and *copAp::lux* fusions were constructed and tested for their ability to indicate the presence of available metals. The purpose of the study was to evaluate the influence of several key environmental and physiological factors that can affect the bioluminescence response. Important variations in light output were found between cells harvested at different growth phases, with maximum luminescence occurring in early stationary phase. Similar results were previously reported with different bacterial strains bearing reporter plasmids containing the full *lux* operon (Rattray et al. 1990; Neilson et al. 1999). Neilson et al. (1999) proposed that the growth phase dependent fluctuations in luminescence could be due to alterations in aldehyde substrate availability. However, the molecular events that lead to maximum light emission when cells enter the stationary phase are still unclear. The medium composition is also an important factor to be considered when creating a new bioassay, particularly when metals are the target compounds. The reduced sensitivity of the *zntAp::lux* biosensor in LB compared to GGM medium is probably due to metal chelation or precipitation by medium components such as inorganic phosphates (Hughes and Poole 1991) or dissolved organic carbon which has been shown to alter the bioavailability of Hg(II) (Barkay et al. 1997). Similar effects of the medium composition on the bioluminescence response of metal sensor strains has also been reported by Tauriainen et al. (1998).

A number of metal ions were found to induce bioluminescence in MG1655(pZNT-lux). In the present study, we showed that Cd(II), Pb(II), Zn(II) and Hg(II) are strong inducers of the luminescence response. Even though induction of *zntA* expression by these four metal ions is not surprising, striking differences were found in the induction levels reported here compared with earlier studies (Babai and Ron 1998; Brocklehurst et al. 1999; Binet and Poole 2000), that may be attributed to differences in the assay conditions and/or the fusion constructs. The regulation of *zntA* expression is mediated by ZntR, a member of the MerR family of transcription regulators, that has been shown to bind the *zntA* promoter and activate transcription in response to Zn(II) (Outten et al. 1999). More recently, Binet and Poole (2000) demonstrated that Cd(II)- and Pb(II)-dependent transcriptional up-regulation of *zntA* is also mediated by ZntR. We report here for the first time that other metal ions, including Co(II), Ni(II), SbO_2^-, CrO_4^{2-} and $Cr_2O_7^{2-}$, significantly induce the *zntAp::lux* fusion. One possible explanation may be that ZntR has a weak affinity and can form an active complex with these metals, as judged by the high concentrations required to trigger the luminescence response of MG1655(pZNT-lux). Results obtained with the *copAp::lux* fusion are consistent with previous observations indicating a specific induction of the *copA* gene by copper and silver ions (Rensing et al. 2000). Expression of *copA* is known to be regulated by CueR, another member of the MerR family, whose interaction with Cu and the *copA* promoter region has been characterized (Outten et al. 2000). At present, however, the molecular basis of metal recognition and signal transduction by both ZntR and CueR remains to be elucidated. Information on the metal binding site of regulators of the MerR family could be exploited to generate reporter strains with new metal specificities for environmental metal monitoring. Alternatively, the concomitant use of

bacterial sensors with different metal response specificities could allow the qualitative detection of each metal in a mixture.

Biologically available concentrations of metals in the environment can be altered by chelating substances from natural (humic and fulvic acids) or anthropogenic sources (EDTA or polyphosphates). Metal solubility can also be altered by microbial processes. For example, sulphide produced by sulphate-reducing bacteria plays an important role in metal sulphide precipitation and immobilization in sediments, and is widely used in bioremediation processes (Gadd 2000). Using two metal ligands, we demonstrated that the sensor strains are able to distinguish bioavailable from total metal content in the assay. The progressive restoration of luminescence of the two strains exposed to toxic concentrations of metal by addition of EDTA supports the hypothesis of metal ion complexation and subsequent reduced availability (and toxicity) to the strain. This observation is consistent with studies by Campbell et al. (2000) and Tauriainen et al. (2000), who used luminescent reporter bacteria to analyse the effect of EDTA on either toxicity or bioavailability of metals. Results with Na_2S showed that sulphide can also affect the bioavailability of metals to *E. coli*, albeit in a less effective manner than chelation with EDTA, presumably by forming insoluble metal sulphides that cannot enter the cell.

Taken together, our results confirm the potential of luciferase gene fusion technology in the monitoring of metal contaminants. The assay is simple, sensitive, gives results in a relatively short time and allows the assessment of the biologically available fraction of metals. This work is a step towards an application of the reporter strains in the analysis of complex metal-contaminated environmental samples.

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