

Improving the sensitivity of bacterial bioreporters for heavy metals

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Whole-cell bacterial bioreporters represent a convenient testing method for quantifying the bioavailability of contaminants in environmental samples. Despite the fact that several bioreporters have been constructed for measuring heavy metals, their application to environmental samples has remained minimal. The major drawbacks of the available bioreporters include a lack of sensitivity and specificity. Here, we report an improvement in the limit of detection of bacterial bioreporters by interfering with the natural metal homeostasis system of the host bacterium. The limit of detection of a *Pseudomonas putida* KT2440-based Zn/Cd/Pb-biosensor was improved by a factor of up to 45 by disrupting four main efflux transporters for Zn/Cd/Pb and thereby causing the metals to accumulate in the cell. The specificity of the bioreporter could be modified by changing the sensor element. A Zn-specific bioreporter was achieved by using the promoter of the *cadA1* gene from *P. putida* as a sensor element. The constructed transporter-deficient *P. putida* reporter strain detected Zn²⁺ concentrations about 50 times lower than that possible with other available Zn-bioreporters. The achieved detection limits were significantly below the permitted limit values for Zn and Pb in water and in soil, allowing for reliable detection of heavy metals in the environment.

Introduction

While chemical and physical methodologies for monitoring environmental pollution can be powerful, accurate and sensitive, they are also costly and require specialized laboratories. In addition, they fail to provide data on the bioavailability of a pollutant and its effects on living systems. Therefore, whole-cell-based bioassays, which are easily applicable and inexpensive, and which report not only on the presence of a chemical, but also on its bioavailability and its biological effects, are being developed. Whole-cell bioreporters are living microorganisms that produce a specific quantifiable output in response to target chemicals. On the basis of detection specificity, bioreporters can be broadly divided into three groups: nonspecific, semi-specific and specific. Nonspecific bioreporters are useful for measuring the toxicity of samples, but do not provide any information about the identity of the contaminants.^{1,2} Recombinant semi-specific bioreporters allow for the detection of compounds that cause certain cellular responses, such as stress³⁻⁵ or DNA damage^{6,7} without further characterizing the compound. Specific bioreporters, first described by King et al.⁸ respond only to a certain compound or class of compounds and therefore permit a quantitative analysis of the contaminant concentration.

In general, specific bacterial bioreporters combine a sensing element, which is responsible for detecting the analyte, and a reporter element, which allows for quantification of the signal.^{9,10}

In this manner, bacterial bioreporters have been developed for heavy metals¹¹ and several organic compounds.^{9,12}

In heavy metal bioreporters, the sensor element usually consists of a specific promoter fused to a reporter gene, and a gene encoding for a transcription factor that in response to certain metal ions activates the promoter. Binding of a metal ion to the transcription factor induces the expression of the reporter gene, thus converting metal sensing into a detectable output signal. The most important characteristics of bacterial bioreporters—specificity (which metals are detected) and sensitivity (what concentrations are detected; characterized by the limit of detection)—depend on the transcription factor used in the sensing element as well as on the microbial metal homeostasis and resistance systems.¹³ In bacteria, homeostasis of essential metals and resistance to toxic heavy metals are maintained with the help of influx and efflux transporters. Importantly, it is the activity of these transporters that determines the intracellular metal concentration that is ultimately available for detection by the bioreporters.

Despite the importance of whole-cell bioreporters in determining the biologically relevant fraction of metals in the environment, they are rarely used for actual environmental analyses. The wider application of bioreporters has been partly hindered by low sensitivity and a lack of specificity. The amount of bioavailable metal in complex matrices can be less than 1%, even in highly contaminated samples.^{14,15} Reliable detection of such low concentrations requires improved detection limits. In addition, the

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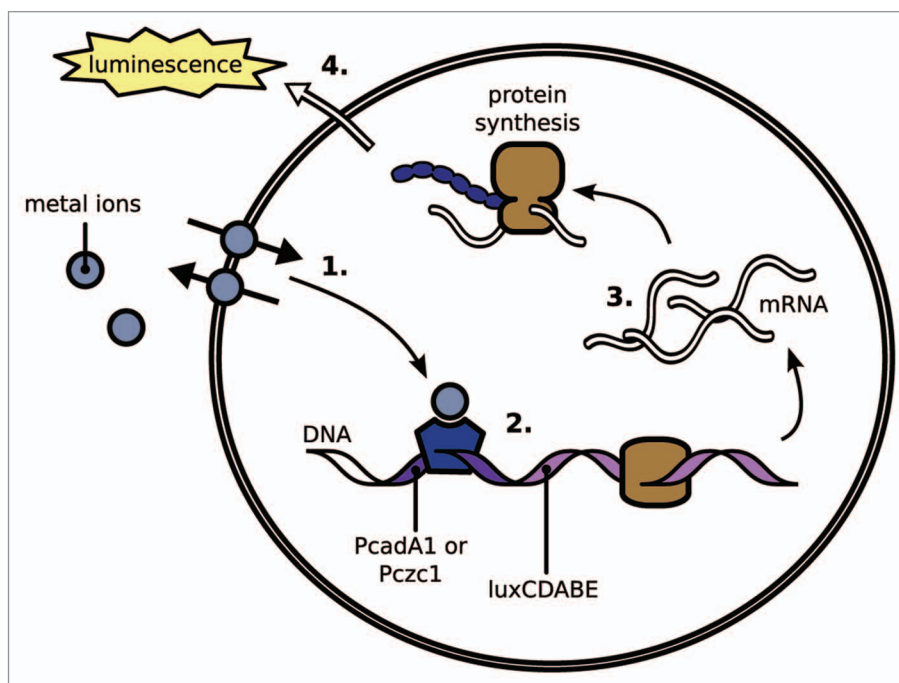


Figure 1. Principle of *Pseudomonas putida* KT2440 and KT2440.2431 metal bioreporters. (1) Uptake and efflux mechanisms regulate the intracellular metal level; in the mutant strain KT2440.2431, the efflux mechanisms have been removed. (2) Upon binding of the metal ion to the transcription factor, transcription is initiated from the PcadA1 or Pczc1 promoter that is fused to the *luxCDABE* operon. (3) mRNA for the reporter proteins is produced and subsequently translated into proteins. (4) The bacterial *lux* operon encodes for luciferase and the proteins necessary for substrate synthesis; therefore, luminescence can be measured directly as no additional substrate is required. Over a certain concentration range, the signal output correlates to the intracellular metal concentration, allowing for quantification of the metal.

ability to specifically characterize the contaminants in a sample requires bioreporters that respond to only one metal.

We hypothesized that, on the removal of specific efflux pumps, bacteria start to accumulate more metal in the cell, and thus, lower extracellular concentrations can be detected. Efflux mechanisms compete with the detection of metal ions and thus lead to a less sensitive response. If all of the metal ions entering the cell were immediately removed, no binding to transcription factors would occur and hence no signal would be generated. However, in the absence of efflux transporters, metal ions accumulate in the cell and the threshold concentration required for the expression of the reporter gene is reached at lower extracellular concentrations. Our approach was to improve the limit of detection (LOD) of *Pseudomonas putida*-based Cd/Zn/Pb-bioreporters by altering the efflux of respective metals from the cytoplasm, thereby causing metal ions to accumulate in the bacterial cell. In *P. putida* KT2440, genes for the divalent heavy metal efflux proteins CadA1, CadA2, CzcA1 and CzcA2 were deleted, and the mutant *P. putida* strain was applied as a Cd/Zn/Pb-bioreporter and tested for improved limit of detection.

Results

Constructed bioreporter strains. *P. putida* strains KT2440,¹⁶ and KT2440.2431,¹⁷ were used as host bacteria. *P. putida* KT2440 is a ubiquitous saprophytic bacterium endowed with a remarkable adaptability to diverse environments and is thus suitable for the

analysis of environmental samples. *P. putida* KT2440.2431 is a metal transporter-deficient mutant of the strain KT2440. In *P. putida* KT2440.2431, genes for four Zn/Cd/Pb efflux transporters (P-type ATPases CadA1 and CadA2 and CBA transporters CzcCBA1 and CzcCBA2) have been disrupted, rendering the cell susceptible to heavy metals. As sensor-reporter elements, we used fusions of heavy-metal-regulated *cadA1* and *czcCBA1* promoters from *P. putida* KT2440 to bacterial bioluminescence operon *luxCDABE* in a low copy number plasmid. Altogether, four different bioreporter strains, KT2440(pDNPczc1lux), KT2440.2431(pDNPczc1lux), KT2440(pDNPcadA1lux) and KT2440.2431(pDNPcadA1lux) (Fig. 1), were tested for the response to heavy metals.

Optimization of the assay. To achieve minimal LOD of the bioreporters within a reasonable testing time, different assay conditions were tested. First, the inducibility of the bioreporters at different growth stages was tested as metal-induced expression from PcadA1 and Pczc1 promoters is known to be dependent on the growth phase of the bacteria.¹⁷ The response of the reporter strains to metals was largely dependent on the growth phase (data not shown). The lowest metal concentrations were detected with bacteria grown to the early exponential phase. Therefore, bacteria grown to the exponential phase ($OD_{600} \sim 0.015 \dots 0.017$ measured on 96-well plate in 200 μ l) were used in further experiments.

In addition, an effect of incubation time (i.e., contact time of the reporter bacteria with the metal) on sensor inducibility

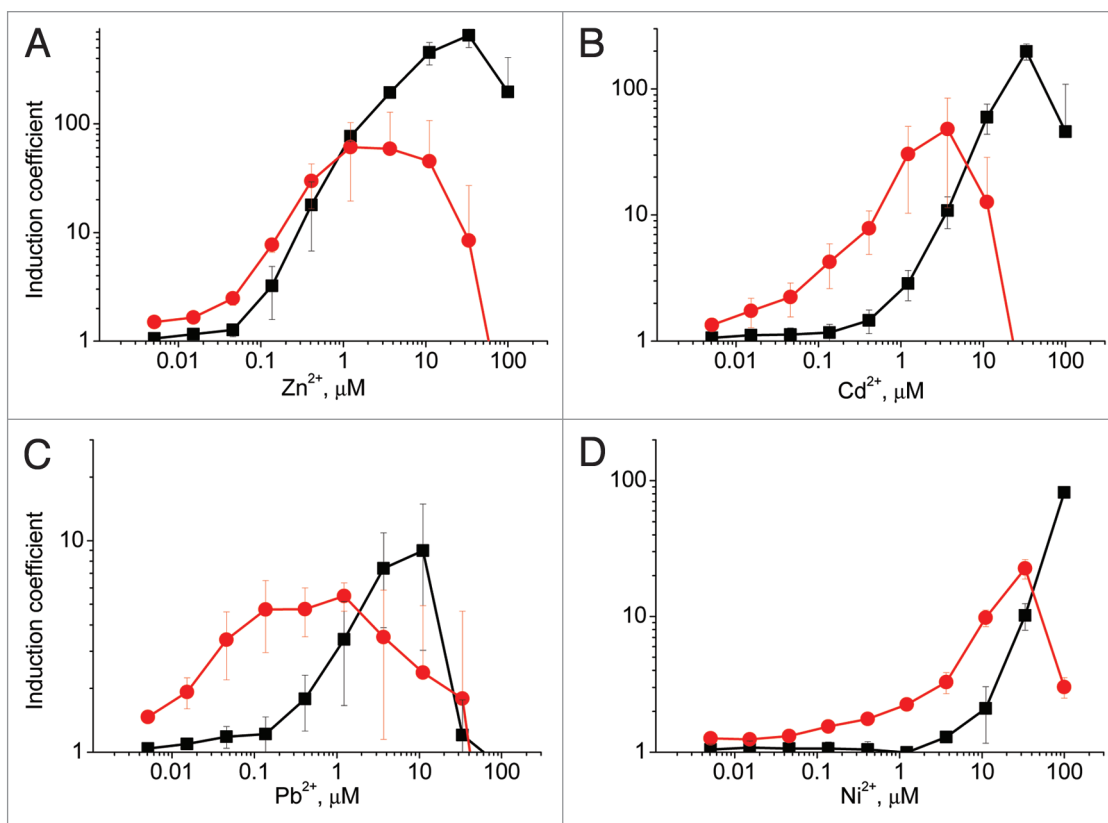


Figure 2. Inducibility of *P. putida* KT2440(pDNPczc1lux) (black squares) and KT2440.2431(pDNPczc1lux) (red circles) with Zn²⁺ (A), Cd²⁺ (B), Pb²⁺ (C) and Ni²⁺ (D). Data represent the mean ± standard deviation of at least three independent experiments.

was investigated. Clear induction was already visible after 1 hour of incubation. For the wild-type reporter strains, further incubation had no effect on the LOD. However, for the mutant reporter strains, up to 10-fold improvement could be achieved by extending the incubation to 3 hours. For example, the LOD of *P. putida* KT2440.2431(pDNPczc1lux) for Cd²⁺ shifted from 0.1 μM to 0.02 μM by extending the incubation from 1 to 3 hours. Although even longer incubation times slightly improved the sensitivity (as low as 0.008 μM LOD for Cd²⁺ could be achieved), a 3-hour incubation time was used in further experiments to balance the time used for the whole assay. The decrease with time in the LOD of the efflux transporter-deficient strain could be explained by the continuous accumulation of metal ions in the cell and the consequent detection of lower extracellular metal concentrations.

Sensitivity and specificity of the reporter strains. The constructed bacterial bioreporters were assayed with different divalent heavy metals to determine their sensitivity (LOD) and specificity (metals detected) using the optimized assay conditions. The Zn/Cd/Pb efflux transporter-deficient strain KT2440.2431 was used in parallel with the wild-type strain KT2440.

As hypothesized, the removal of efflux transporters affected the LOD of the bioreporters. *P. putida* KT2440.2431(pDNPczc1lux) could detect Pb²⁺ at 45-fold, Cd²⁺ at 12-fold, Ni²⁺ at 10-fold and Zn²⁺ at 3-fold lower concentrations than its wild-type counterpart KT2440(pDNPczc1lux) (Fig. 2 and Table 1). For the

wild-type strain, Zn²⁺ was the best inducer, whereas for the mutant strain, Zn²⁺, Cd²⁺ and Pb²⁺ were detected at the same concentrations. For both strains, Ni²⁺ was a poor inducer as 100-fold higher concentrations were needed for induction. No induction was observed with Hg²⁺ or Co²⁺. Toxic concentrations causing a rapid decrease in the luminescence signal were much lower for the mutant strain (Fig. 2), indicating that metals accumulated in this strain.

Although substantial improvements in LODs were achieved by using the promoter of the *czcCBA1* operon as a sensor element, the lack of specificity still remained a problem. Therefore, we tested a promoter of the *cadA1* gene, which is known to be induced by Zn²⁺ and slightly by Cd²⁺, but not by other metals,¹⁷ as a sensor element. *P. putida* KT2440(pDNPcadA1lux) was inducible only with Zn²⁺, and expectedly, the mutant strain KT2440.2431(pDNPcadA1lux) could detect about eight-fold lower Zn²⁺ concentrations than the wild-type strain (Fig. 3 and Table 1). Although *P. putida* KT2440.2431(pDNPcadA1lux) could not detect concentrations as low as KT2440.2431(pDNPczc1lux), the great advantage of the reporter strain harboring the promoter of *cadA1* as a sensor element was its specificity—no metals other than Zn²⁺ induced this reporter strain.

Performance of the bioreporters in Zn-contaminated soil samples. The performance of the constructed bioreporter strains was tested on soil samples intentionally contaminated with a known amount of Zn²⁺. In the measurements of undiluted soil

Table 1. Limits of detection of Zn-, Cd- and Pb-sensing bioluminescent bacterial bioreporters

Bacterial bioreporter	Limit of detection, μM			Reference
	Zn ²⁺	Cd ²⁺	Pb ²⁺	
<i>P. fluorescens</i> OS8::KncadR _{Pcad} Alux ^a	4	0.03	0.3	28
<i>E. coli</i> MC1061(pSLzntR/pDNPzntAlux) ^a	5	0.01	0.7	28
<i>E. coli</i> MC1061(pzntRluc) ^b	40	0.06	n.t.	29
<i>E. coli</i> MG1655(pZNT-lux) ^b	1	0.01	0.03	30
<i>S. aureus</i> RN4220(pTO024) ^c	1	0.01	0.03	31
<i>B. subtilis</i> BR151(pTO024) ^c	1	0.03	n.i.	31
<i>P. putida</i> KT2440(pDNPczc1lux) ^d	0.16 (0.08)	1.12 (0.49)	0.90 (0.41)	This study
<i>P. putida</i> KT2440.2431(pDNPczc1lux) ^d	0.05 (0.02)	0.09 (0.05)	0.02 (0.02)	This study
<i>P. putida</i> KT2440(pDNPcadA1lux) ^d	0.89 (0.63)	n.i.	n.i.	This study
<i>P. putida</i> KT2440.2431(pDNPcadA1lux) ^d	0.12 (0.08)	n.i.	n.i.	This study

^alimit of detection (LOD) determined as a concentration that caused $2 + 6 \text{ SD}_w/L_w$ -fold induction. ^bLOD determined as a concentration that caused two-fold induction. ^cLOD determined as a concentration that caused $L_w + 2 \text{ SD}_w$ -fold induction. ^dLOD determined as a concentration that caused $(1 + 2 \text{ CV}_w)/(1 - 2 \text{ CV}_w)$ -fold induction; upper estimate of LOD (confidence level 97.7%) and mean LOD (in parenthesis) are presented (see Materials and Methods for details). L_w , luminescence in water; SD_w , standard deviation of the signal measured in water; CV_w , coefficient of variation of the signal measured in water; n.t., not tested; n.i., not inducible.

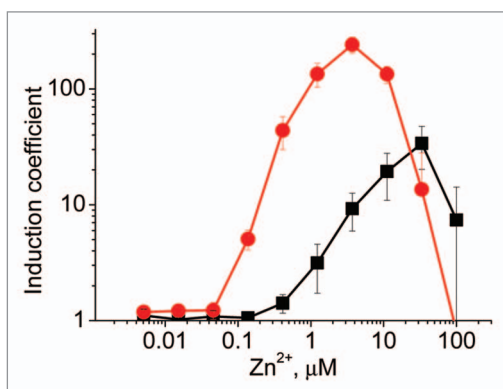


Figure 3. Inducibility of *P. putida* KT2440(pDNPcadA1lux) (black squares) and KT2440.2431(pDNPcadA1lux) (red circles) with Zn²⁺. Data represent the mean \pm standard deviation of three independent experiments.

samples wild-type *P. putida* KT2440(pDNPcadA1lux) could reliably detect zinc from the soil that contained 65 mg/kg of Zn²⁺; whereas, the more sensitive transporter-deficient strain KT2440.2431(pDNPcadA1lux) could detect as low concentrations as 20 mg/kg of Zn²⁺ (Fig. 4A).

In highly contaminated samples metal concentrations become toxic to the cell and measurements of undiluted samples do not give correct estimation of the bioavailable amount of the metal. In such cases additional diluting of the sample is required in order to reach concentrations which are close to the LOD of the bioreporter. The transporter-deficient reporter strain KT2440.2431(pDNPcadA1lux) could detect about 10-fold lower Zn concentrations than the wild-type strain KT2440(pDNPcadA1lux) in the dilution series of soil spiked with 654 mg/kg of Zn²⁺ (Fig. 4B). Thus, as was the case with standard metal solutions, transporter-deficient bioreporter performed better also in actual environmental samples.

Discussion

Dozens of bioreporters have been constructed, and their suitability for testing environmental samples has been demonstrated.¹³ Despite this research, bioreporters are not yet widely used in environmental applications, partly due to their high LODs and lack of specificity. We approached the sensitivity and specificity issues by disrupting the metal homeostasis of the host bacteria and testing different sensor elements.

In this study, we constructed new transporter-deficient bacterial bioreporter strains *P. putida* KT2440.2431(pDNPczc1lux) and KT2440.2431(pDNPcadA1lux), which, depending on the metal, could detect 3–45-fold lower metal concentrations as compared to their wild-type counterparts. The improvement in LODs was achieved due to the disruption of metal efflux transporters and consequent accumulation of metals in the cell. *P. putida* KT2440.2431(pDNPczc1lux), whose upper estimate of LOD for zinc was 0.05 μM (3.3 $\mu\text{g/l}$), is the most sensitive bacterial sensor for zinc constructed thus far (Table 1). Although the LOD for lead and cadmium remained on the same level as those for already existing bioreporters (Table 1), the success of metal homeostasis interruption indicates that improved LOD for these metals could also be achieved by modifying already existing bioreporter strains. A similar strategy has been shown to work for Cu/Ag-bioreporters, but not for As-bioreporters. The removal of CopA, a Cu/Ag-exporting ATPase, from *E. coli* rendered a bioreporter employing the *copA* promoter as a sensor system 15-fold more sensitive to Cu and eight-fold more sensitive to Ag.¹⁸ On the other hand, Tauriainen et al.¹⁹ compared the performance of two *E. coli* As-bioreporters—one with an intact As detoxification system and another containing a deletion in the chromosomal arsenite-resistance operon. They found no differences in performance of these bioreporters, showing that the lack of the *ars* operon did not cause a remarkable accumulation of As in the cell.

Almost all bioreporters constructed to date detect only intracellular metal. In addition to causing the accumulation of metals

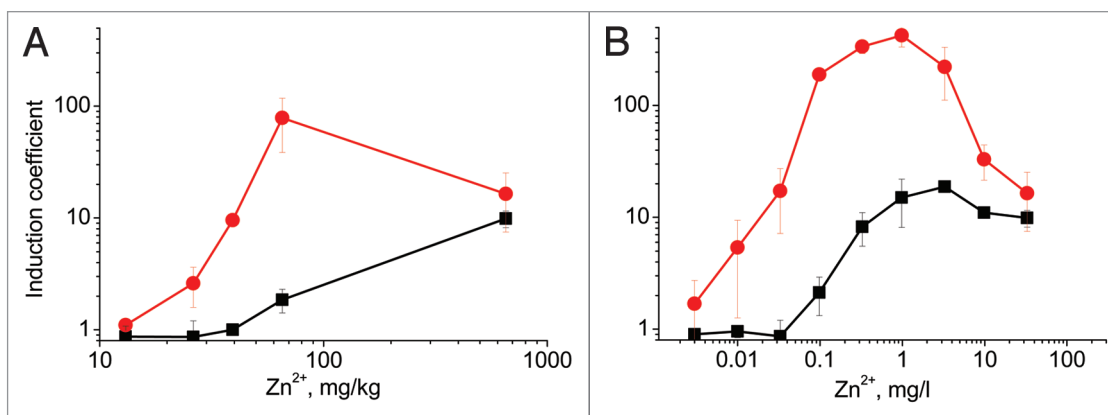


Figure 4. Performance of *P. putida* KT2440(pDNPcadA1lux) (black squares) and KT2440.2431(pDNPcadA1lux) (red circles) in Zn-contaminated soil samples. (A) Induction of the bioreporters as a function of Zn²⁺ concentration in the soil samples. The x-axis shows the spiked Zn concentration in the soil. Each measurement point corresponds to a soil sample spiked with different concentration of Zn. (B) Induction of the bioreporters in the dilution series of the soil sample spiked with 654 mg/kg of Zn²⁺. The x-axis shows the actual Zn concentration in the diluted samples. Data represent the mean \pm standard deviation of three independent experiments.

in the cell by removing efflux transporters, permeabilization of the membrane or additional metal uptake mechanisms could also improve the LOD of bacterial bioreporters. The influence of uptake transporters has been tested on a Hg-bioreporter.²⁰ In addition to detoxification proteins, the Hg-resistance operon encodes for a protein that mediates the uptake of Hg²⁺ ions into the cell. Employing this protein in the bioreporter construct improved the sensitivity of the Hg-bioreporter two-fold.²⁰ Although specific uptake systems only exist for a limited number of metals, the proteins responsible for the uptake of most metals are known. Overexpression of these systems in combination with the removal of efflux transporters would thus be one approach to improving the LOD of bacterial bioreporters.

In addition to improving the LOD of Zn/Cd/Pb-bioreporters, we aimed at more specific reporters by changing the sensor element. *P. putida* KT2440.2431(pDNPcadA1lux), which carried the *cadA1* promoter as a sensor element, proved to be Zn²⁺-specific. Although *P. putida* KT2440.2431(pDNPcadA1lux) was somewhat less sensitive than *P. putida* KT2440.2431(pDNPczc1lux) (Table 1), its specificity is a great advantage over other bioreporters. Restricted specificity allows for more accurate quantification of a certain contaminant regardless of other contaminants in the sample. The existence of a Zn-specific sensor element is peculiar, as all of the transcription factors and resistance mechanisms known for Zn²⁺ thus far cross-react with Cd²⁺ and Pb²⁺ (see Table 1).^{17,21-24} However, as only three sensor elements (the promoters of *zntA* from *E. coli*, *cadA* from *P. putida* and *cadA* from *S. aureus*) have been previously used for the construction of Zn/Cd/Pb-bioreporters, it is still possible that an open-minded search for new heavy-metal-regulated promoters would yield bioreporters with different specificities.

Although the LOD is an important characteristic of bacterial bioreporters, no common method for calculating the LOD has been established in the literature (see Table 1). Several of the methods applied for calculating the LOD lack any statistical basis. However, as bacterial bioreporters are living systems, some variability in the signal within one experiment and between

Table 2. Limit values of zinc, cadmium and lead in the European Union, compared to the upper estimates of the limit of detection of the bacterial bioreporter *P. putida* KT2440.2431(pDNPczc1lux)

Metal	Limit value		Upper estimate of the limit of detection, mg/l
	Surface water, mg/l ^a	Soil, mg/kg ^b	
Zn	3	150...300	0.003
Cd	0.005	1...3	0.010
Pb	0.05	50...300	0.004

^aCouncil Directive 75/440/EEC; ^bCouncil Directive 86/278/EEC.

independent experiments is natural. Therefore, a statistically sound method for determining LOD should be used. IUPAC suggests that LOD for analytical methods is determined as the concentration which has a response equal to the mean response plus two or three standard deviations of the blank samples depending on the confidence level desired.²⁶ However, this method eliminates only the false positives and does not take into account the variability in the signal measured for the analyte. We used here the method recommended for immunoassays.²⁷ This method considers the variability in the signal for both the blank and also the analyte. The concentration of the analyte where the confidence interval does not overlap with the confidence interval of the blank is considered as the LOD.²⁷ Thus, the method eliminates both the false positives and the false negatives. In addition to considering the variation within the experiment we took into account the variability between the independent experiments and reported the upper estimate of LOD in addition to reporting the mean LOD. If only the mean LOD is used for characterizing the bioreporter, 50% of the experiments actually have a higher LOD than initially reported. However, the upper estimate of LOD guarantees that with a probability of 97.7% the LOD in a single experiment is below the reported level. Therefore, it is important to consider the variability of the method when reporting the characteristics of the bioreporter.

The new bioreporters constructed in this work, *P. putida* KT2440.2431(pDNPCzc1lux) and KT2440.2431-(pDNPCadA1lux), are suitable for measuring zinc, cadmium or lead from environmental samples, as their upper estimate of LOD remained well below the limit values of water and soil contaminants set by the European Union (Table 2). Our results show that the limit of detection of bacterial heavy metal bioreporters could be improved by removing heavy metal efflux pumps, thereby artificially increasing the intracellular concentration of the metal. This study indicates that the disruption of metal homeostasis in bacteria is a feasible approach for the development of bacterial bioreporters with increased sensitivity. The use of hyper-sensitive bioreporters in environmental risk assessment opens the possibility for the detection of low-level pollution.

Materials and Methods

Bacterial strains and growth conditions. As bioreporters, *P. putida* strains KT2440,¹⁶ and KT2440.2431,¹⁷ were used. Both strains were transformed with the sensor plasmids pDNPCadA1lux and pDNPCzc1lux.¹⁷ These plasmids contain a *luxCD-ABE* operon from *Photobacterium luminescens* under the control of the *cadA1* and *czcCBA1* promoters from *P. putida*, respectively.

Bacteria were grown in a heavy metal MOPS (HMM) medium²⁵ supplemented with 0.05% caseine hydrolysate, 0.4% glucose and 12 µg/ml tetracycline. For maintaining the strains, HMM plates containing 1.5% agar were used.

Induction assay. Inducibility of the constructed strains was tested with different heavy metals. Solutions of CdCl₂ (stock solution 1 M), ZnCl₂ (0.1 M), HgCl₂ (0.1 M), NiSO₄ (1 M), CoCl₂ (1 M) and Pb (CH₃COO)₂ (0.05 M) at different concentrations were prepared in MilliQ water (Millipore, France). First, metal dilutions were prepared with a Biomek NX^P (Beckman Coulter, Fullerton) robot using a dilution factor of 1:3. Then, 20 µl of the metal dilution was dispensed to a white 384-well plate (Thermo LabSystems, Finland), and an equal amount of bacterial suspension was added using a Biomek NX^P pipetting robot. The plates were incubated for 3 h at 30°C, and the luminescence was measured with a Victor Wallac 1420 multilabel counter (PerkinElmer, Finland). The samples in every assay were analyzed in four parallels, i.e., each metal concentration was plated in four wells. At least three independent assays were performed for every strain. Induction of the bioreporters was reported as an induction coefficient (IC), calculated as follows: $IC = L_M/L_w$, where L_M is the luminescence in the metal solution and L_w is the background luminescence measured in MilliQ water.

Testing conditions. The growth and test conditions were optimized to achieve a satisfactory limit of detection within a minimal time. The cells were grown overnight in HMM medium, diluted 100-fold in fresh medium and then grown at 30°C with shaking. Aliquots of bacteria were removed at different time points and used in the induction assays. Growth curves were determined by measuring the OD₆₀₀ values at 1-h intervals, and growth phases were assigned according to different stages of the growth curve. OD₆₀₀ was measured with Victor Wallac 1420 on 96-well plate in 200 µl.

The optimal incubation time was determined by measuring induction plates at 1-h intervals.

Determination of the limit of detection. The limit of detection for bacterial bioreporters was determined as the metal concentration which induced the reporter to above the threshold level LOD_{IC}, calculated according to the equation $LOD_{IC} = (1 + 2 CV_w)/(1 - 2 CV_w)$. Here CV_w is coefficient of variation of the signal determined in water (background or blank). The signal in water was always measured in at least 12 parallels, but as it was not reasonable to measure samples in as many parallels it was assumed that CV for samples is the same as for water. This equation assures that, within the confidence interval of 97.7% the lowest signal from the sample, $L_M - 2 SD_M$, is higher than the highest value for the blank within the same confidence interval, $L_w + 2 SD_w$, thus eliminating both false positives and false negatives. The lowest detectable concentration was determined separately for each measurement (according to the LOD_{IC} of each independent measurement). An upper estimate of LOD was determined as the mean plus two standard deviations of the mean, calculated from the independent measurements. This corresponds to a 97.7% confidence level, i.e., the probability that the LOD determined from a single experiment is below this upper limit is 97.7%. In order to facilitate the comparison to previously published bioreporters also the mean LOD was presented.

Analyses of the soil samples. Artificial Zn-contaminated soil samples were made by spiking industrially produced garden soil (Rainbow, Finland) with different Zn²⁺ concentrations. One gram of soil (dry weight) was mixed with 9 ml of ZnCl₂ solution (final concentrations ranging from 6.54 to 654 mg of Zn²⁺ per 1 kg of soil) and then shaken on vortex for 1 h. Non-spiked soil sample (MilliQ water added instead of ZnCl₂ solution) was used as a background control to take into account potential changes in the luminescence not caused by zinc. This includes potential interference of the sample matrix (stimulation or quenching of the luminescence) due to the presence of nutrients, toxicants or turbidity of the sample, and changes in the luminescence due to the changes in the physiological state of the cell. Samples were analyzed immediately and stored for further measurements at -20°C.

For the induction assay 50 µl of the soil suspension was plated into a 96-well microplate (Thermo LabSystems, Finland) and mixed with an equal volume of a bacterial suspension at an early exponential growth phase (OD₆₀₀ ~ 0.015...0.020). Plates were then incubated for 3 h at 30°C, and luminescence was measured with a Victor Wallac 1420 multilabel counter. All measurements were obtained using two replicates in three independent experiments. Sample specific IC was calculated by dividing luminescence measured in spiked sample with luminescence measured in non-spiked sample at respective dilution. The soil sample was considered to induce the bioreporter if higher IC than LOD_{IC} (see above) was achieved.

Note that the measurement protocol causes 20-fold dilution of the initial sample (10-fold in preparation of suspension and additional two-fold in actual measurement). The soil suspensions which induced the bioreporters were further diluted up to 10,000-fold with MilliQ water in order to achieve IC close to LOD_{IC}.

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