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ORIGINAL PAPER

A multi-channel bioluminescent bacterial biosensor for the on-line detection of metals and toxicity. Part I: design and optimization of bioluminescent bacterial strains

Thomas Charrier · Marie-José Durand · Sulivan Jouanneau · Michel Dion · Mimma Pernetti · Denis Poncelet · Gérald Thouand

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Abstract This study describes the construction of inducible bioluminescent strains via genetic engineering along with their characterization and optimization in the detection of heavy metals. Firstly, a preliminary comparative study enabled us to select a suitable carbon substrate from pyruvate, glucose, citrate, diluted Luria–Bertani, and acetate. The latter carbon source provided the best induction ratios for comparison. Results showed that the three constructed inducible strains, *Escherichia coli* DH1 pBzntlux, pBarslux, and pBcoplux, were usable when conducting a bioassay after a 14-h overnight culture at 30 °C. Utilizing these sensors gave a range of 12 detected heavy metals including several cross-detections. Detection limits for each metal were often close to and sometimes lower than the European standards for water pollution. Finally, in order to maintain sensitive bacteria within the future biosensor-measuring cell, the agarose immobilization matrix was compared to polyvinyl alcohol (PVA). Agarose was selected because the detection limits of the bioluminescent strains were not affected, in contrast to PVA. Specific detection and cross-detection ranges determined in this study will form the basis of a multiple metals detection system by the new multi-channel Lumisens3 biosensor.

Keywords Bioluminescence · Heavy metals · Bacteria · Detection · Immobilization

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T. Charrier · M.-J. Durand · S. Jouanneau · G. Thouand (✉)
Nantes University, PRES UNAM, Campus de la Courtaisière-IUT,
UMR CNRS 6144 GEPEA, CBAC,
18 Bd Gaston Defferre,
85035 La Roche-sur-Yon cedex, France
e-mail: gerald.thouand@univ-nantes.fr

M. Dion
Nantes University, PRES UNAM,
Biotechnologie, Biocatalyse, Biorégulation,
UMR CNRS 6204,
2, Rue de la Houssinière,
BP 92208, 44322 Nantes cedex 3, France

M. Pernetti · D. Poncelet
ONIRIS-ENITIAA, UMR CNRS GEPEA,
Rue de la Géraudière,
BP 82225, 44332 Nantes cedex 3, France

Introduction

In 2001, the European Community published a list of priority substances to be detected in water; this list mentioned heavy metals such as cadmium, lead, and mercury. Heavy metals are naturally present in water at the nanogram per liter level, but anthropogenic activities increase this level [1]. Because of their considerable solubility in water, heavy metals are easily absorbed by living organisms and accumulate at the end of the food chain [2]. Indeed, industrial wastes are mainly responsible for the current heavy metal pollution of rivers [3]. These wastes are usually collected and treated by the Urban Waste Water Treatment Plants network which, according to Karvelas et al. [4], rejects between 37% and 77% of the metals.

Heavy metals such as lead, mercury, cadmium, and arsenic constitute a significant potential threat to human health. The toxicity of metals most commonly involves the brain and the kidney, but other manifestations occur, and

some metals, such as arsenic, are clearly capable of causing cancer [5, 6].

From a general point of view, the action of heavy metals in the bacterial cytoplasm causes important oxidative stresses [7]. Heavy metals enter the bacterial cell via the regulation systems of divalent cations or oxyanions (i.e., SO_4^{2-}, HPO_4^{2-}, Fe^{2+}, Mg^{2+}, and Mn^{2+}) without any differentiation. To cope with this passive absorption, bacteria have developed two kinds of resistance systems: a nonspecific system linked to the concentration gradient and a more specific and inducible system activated by metals that have been divided into three families and from which the current heavy metal-sensitive bacteria are constructed (the ATPase pump–CPx-type ATPase; the complexation systems played by glutathione, for example, and the reduction mechanism for oxyanion, for a review, see [8]). It is important to note that the overall resistance of the bacteria to heavy metals is a complex combination of activation involving several mechanisms. The inducible resistance mechanisms are thus not activated by only one metal, even if their name would seem to imply this [8]. In the same way, only one metal can activate several systems of resistance [9, 10].

The analytical methods currently used to quantify metals are atomic absorption or furnace atomic absorption and atomic emission. These methods are very sensitive (limit of detection for cadmium, 1 ppb) but require an important pretreatment of samples [11]. In addition, detection becomes more complex because elements contained in the organic matrices are able to disturb the atomization step [12].

To cope with the need for fast and simple methods, metal detection bioassays were developed. The use of the reporter bioluminescence reaction producing light at 490 and 590 nm [13, 14] after a complex biochemical reaction [15] was first described in 1993 for Hg detection and remains a standard today [16]. Bioluminescence is the light produced by some organisms mediated by the enzyme luciferase or a photoprotein mediated an oxidation reaction [17]. A reporter gene is fused to another gene or a promoter so that the expression of that gene or promoter may be assayed. The product of the reporter gene is typically more stable and easier to detect than the gene to which it is fused [18]. The strong application potential of this bioassay led to several other genetic constructions for most of the metals [16, 19–32]. The detection limit ranges from nanomolars to several hundred micromolars with time of detection from 30 min to several hours depending on the metal, the host strain, and the promoter (see Magrisso et al. [33] for a comprehensive review).

The choice of heavy metal resistance systems in metal detection depends on the range of detected metals and on the associated detection limits. Indeed, a bacterial biosensor must be able to detect heavy metal concentrations that are close to established standards. Therefore, the choice of an inducible resistance system when building a sensitive strain is primarily motivated by its sensitivity to metal in real condition. According to the regulation modes of the heavy metals resistance system, two kinds of mechanisms can be distinguished: MerR mechanisms and SmtB/ArsR mechanisms. MerR-like regulators are activators/repressor proteins. Regulator proteins sensitive to heavy metals form only one class of this family [34]. In the case of SmtB/ArsR mechanisms, the regulator acts solely as a repressor on the targeted promoter. The binding of the ligand (heavy metal for example) changes the regulator conformation and its affinity towards the promoter's operator sequence. The regulator then leaves the promoter and allows it to become accessible to an RNA polymerase [35–37].

To deal with the multi-sensitive aspect of the bacterial construction, Hakkila et al. [38] designed a bundle of 22 optical fibers bioassay with several heavy metal-inducible bacteria in order to expand the spectrum of metal detection. The authors pointed out the difficulty of detection due to cross-reaction.

Our current work is devoted to the development of a biosensor for the on-line detection of heavy metals by keeping in mind the use of the cross-reaction drawback as a tool to distinguish between metals. The work is divided into two parts: part 1 describes the construction of the bacteria, and part 2 presents the parameters that should be considered for application to the biosensor (Lumisens3).

Experimental

Bacterial strains, media, and growth conditions

A set of four bacteria, namely, *Escherichia coli* pBfilux, *E. coli* Zntlux, *E. coli* Coplux, and *E. coli* Arslux, was used in this study (Table 1). *E. coli* Coplux and *E. coli* Arslux were specifically built for this experimental study, while the other plasmids are coming from previous constructions summarized below [39].

One plasmid was built to allow constitutive expression of bioluminescence, the signal of which decreases when the pollutant concentration is toxic, and three other plasmids were built for heavy metal detection. All of these constructions were transformed in *E. coli* strains.

For molecular biology applications, bacteria were grown in Luria–Bertani (LB) medium supplemented with ampicillin (100 $\mu g mL^{-1}$, Sigma). Solid LB was supplemented with 15 $g L^{-1}$ of bacteriological agar (Biokar Diagnostics).

Culture media for detection with bacteria were prepared with a C/N/P ratio of 100/10/1 (*w*/*w*/*w*) as follows [40]: 1 L of tap water was filtered through a 0.45-μm filter (Sartorius) and

Table 1 Strains and plasmids built or used in this study

Strain (genotype)		Plasmid	Description	References
E. coli XL1 blue	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 *lac*$^-$ F′ [*proAB*$^+$ *lacI*^{q} *lacZ* ΔM15 Tn10 (Tet^r)]	pBtac2	Ap^R, p*tac* promoter, constitutive expression vector	Roche
		pBfiluxCDABE	Ap^R, *ptac*::*luxCDABE*, constitutive expression of the *V. fischeri* bioluminescence *lux* operon	[39]
E. coli DH5α	F$^-$, *lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk$^-$, mk$^+$), *phoA*, *supE*44, λ$^-$, *thi*-1, *gyrA*96, *relA*1	pRL1114	Km^R, containing *luxCDABE* operon from *V. fischeri*	[41]
E. coli DH1	F$^-$, *recA*1 *endA*1 *gyrA*96 *thi*-1 hsdR17 *supE*44 *relA*1	pBarslux	Ap^R, *parsR*::*arsR*::*luxCDABE*, bioluminescent sensor inducible by arsenic	This study
		pBzntlux	Ap^R, *pzntA*::*luxCDABE*, bioluminescent sensor inducible by cadmium and mercury	[39]
		pBcoplux	Ap^R, *pcopA*::*luxCDABE*, bioluminescent sensor inducible by copper	This study

supplemented with the carbon source 1.376 g α-D-glucose monohydrate (Merck) or 2.834 g sodium acetate trihydrate (Panreac) or 1.525 g sodium pyruvate (Sigma) or 2.037 g trisodium citrate (Labosi, France), 1.1919 g NH_4Cl (Merck), 0.028 g K_2HPO_4 (Merck), 5 g NaCl, 0.5 g yeast extract (Merck), and 0.1 g tryptone (Biokar Diagnotics). The pH was adjusted to 7, and the medium was sterilized by autoclaving at 100 °C for 30 min. This media was used for bioassay studies with all the inducible strains.

Batch cultures were grown at 30 °C and shaken at 250 rpm in 100 mL Erlen flasks. Bacterial growth was monitored using a spectrophotometer (Unicam) at an absorbance of 620 nm.

Molecular biology

All oligonucleotide sequences used for polymerase chain reaction (PCR) applications are listed in Table 2.

The *Vibrio fischeri luxCDABE* operon was amplified from plasmid pRL1114 [41] with Dynazyme polymerase (Finnzyme). The PCR fragment was purified (QIAquick kit, Qiagen), digested by the *Nru*I and *Eco*RI restriction enzyme (NEB), and introduced (T4 DNA ligase, NEB) into the pBtac2 vector (Roche), under the control of the p*tac* promoter (constitutive promoter without *lacI* in multicopy), to obtain the plasmid pBfiluxCDABE. This plasmid, when transformed in *E. coli* DH1, allowed a constitutive expression of bioluminescence.

The heavy metal-inducible promoters, *pzntA*, *pcopA*, and *parsR*::*arsrR*, were all amplified on a boiled preparation of *E. coli* DH1 by PCR using Deep Vent polymerase (NEB). Plasmid pBfiluxCDABE was digested by NruI and EcoRI, purified, and then each promoter was cloned into the linearized vector to obtain pBzntlux, pBcoplux, and pBarslux plasmids as shown in Fig. 1. Plasmids pBzntlux, pBarslux, and pBcoplux were transformed in *E. coli* DH1.

Chemicals

The metals used in this study, with a purity greater than 95%, were $CdCl_2$ (Panreac), $HgCl_2$ (Prolabo France), $PbCH_3CH_2O$ (Panreac), $SnCl_2$ (Merck), $ZnCl_2$ (Merck), $NiSO_4$ (Labosi), $CoCl_2$ (Labosi), $K_2Cr_2O_7$ (Labosi), $CrCl_3$ (Acros), $AgNO_3$ (Prolabo, France), $CuSO_4$ (Labosi), $FeSO_4$ (Labosi), $MnCl_2$ (Panreac), and As_2O_3 (Sigma). Metal stock solutions were prepared at a high concentration (1 or 0.5 M) in acidified distilled water (pH≈5–6) and stored at +4 °C in brown bottles for a period of 6 months. Working dilutions of metals were made daily in distilled water acidified to pH≈5–6 and then added to the samples at the appropriate concentration.

Bacterial cells immobilization procedure

Agarose hydrogel matrix and polyvinyl alcohol (PVA) were used for cell immobilization assays according to previously published results [42–44]. For agarose immobilization, strains were cultivated in acetate medium at 30 °C for 14 h. $OD_{620\ nm}$ of the culture was measured, and the cells were diluted with fresh medium to $OD_{620\ nm}$=0.2. At the same time, a 4% agarose solution was prepared by dissolving 0.4 g agarose LGT (Agarose Type VII-A Low Gelling Temperature 26 °C; Sigma, reference A0701) at 90 °C in 10 mL of acetate medium on a warming magnetic stirrer. Five milliliters of this solution was preserved at 37 °C in a water bath with constant agitation. Then, 5 mL of the bacterial solution at $OD_{620\ nm}$=0.2 was added to the 5-mL 4% agarose solution preserved at 37 °C. After homogenization, the final solution reached $OD_{620\ nm}$=0.1 for a final agarose concentration of 2%. As a final step, 100 μL of this solution was poured to each well of a microtiter plate, and the agarose-immobilized cells were used immediately in the bioassay.

For PVA preparation, 10 g of PVA (Sigma) was added to 100 mL acetate medium (10% *w*/*w*) by heating the solution at 80 °C under constant agitation with a magnetic stirrer for

Table 2 Primer sequences and associated PCR products used in this study

PCR product	Oligo's sequence	Name
luxCDABE V. fischeri	ACG TGA ATT CAT GAA TAA ATG TAT TCC AAT GA	luxCDABEvfEco
	ACG TCT GCA GTT AAT CCT TTA TAT TCT TTT GTA TG	luxCDABEvfPst
pcopA E. coli DH1	ATG CTC GCG ACA TTT TGT CCG CCG TTA AGT	pcopAANru
	ATG CGA ATT CCA CTC CTT TAA GAC AGT TTT GAC TG	pcopABEco
parsR::*arsR E. coli* DH1	ATG CTC GCG ATT CCT CTG CAC TTA CAC ATT CG	parsRNru
	ACG TGA ATT CAT CCT CCC GGA TAA AAC ACA TCT	parsREco
pzntA E. coli DH1	CAC TTC CTG ATC GTC CGC TCG CTG CT	pzntA
	AGC ATG AAT TCG GCA TCC TCC GGT TAA GTT T	pzntAEco

Underlined sequences correspond to restriction sites added by PCR

1.5 h. When the solution was perfectly clear, 4.4 g of pure glycerol (Sigma, purity=98%) was added to 40 g of the above PVA solution. The preparation was then mixed for 12 h. For the purposes of immobilization, a volume of the bacterial overnight culture was centrifuged at 5,000×*g* for 10 min. The supernatant was withdrawn, and 10 mL of the immobilization solution (PVA 10%, glycerol 10%) was added to the pellet in order to reach a final $OD_{620\ nm}$=0.1. After homogenization of the suspension, 100 μL was poured in the wells of a microtiter plate using a micropipette with positive displacement (Gilson). The plate was sealed and incubated, first for 1 h at 4 °C and then 16 h at −20 °C for PVA polymerization.

Bioassays

For heavy metal detection, an overnight culture at 30 °C of the inducible bacterial cells was diluted to $OD_{620\ nm}$=0.05

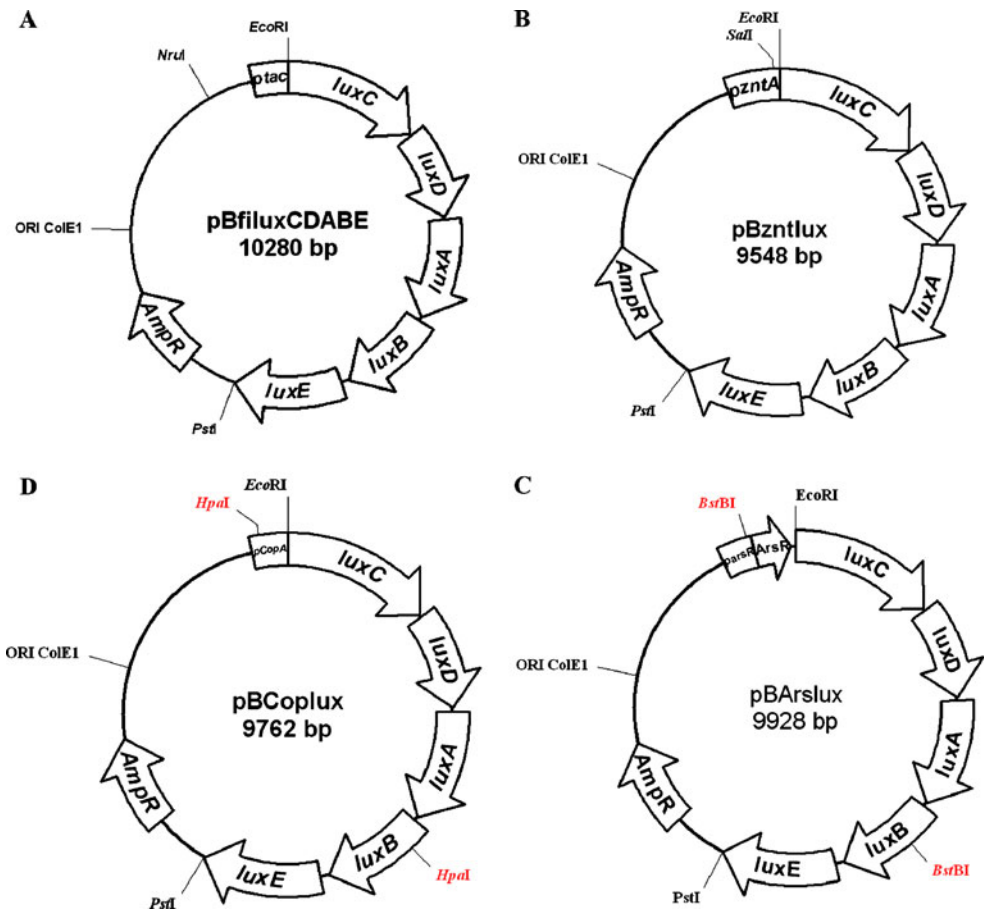


Fig. 1 Maps of plasmids pBfiluxCDABE (**a**), pBzntlux (**b**), pBarslux (**c**), and pBcoplux (**d**) built for heavy metal detection in *E. coli*

in fresh medium in every experiments. One hundred microliters of the dilution were added to a 96-well white microtiter plate. Bacterial bioluminescence was monitored after the addition of 25 μL of heavy metal solutions, the concentration of which ranged from 0.005 to 50 μM. The bioluminescence peak expressed in relative light units per second was recorded, and the induction ratio (IR) was calculated as follows: $IR=(RLUs^{-1})_i/(RLUs^{-1})_o$ where $(RLUs^{-1})_i$ is the bioluminescence after induction with a metal, and $(RLUs^{-1})_o$ is the background bioluminescence (blank without metal).

Bioluminescence measurements were performed at a controlled temperature (30 °C) using a microtiter plate luminometer (Microlumate L96V, EGG Berthold).

Results and discussion

In our study, we chose to work with two MerR mechanisms: ZntA/ZntR and CopA/CopR, and the ArsR mechanism of the SmtB/ArsR system previously used in sensitive bioluminescent strains [23, 24, 29, 32].

For MerR system constructions, only the inducible promoters, *pzntA* or *pcopA*, were cloned upstream of the *lux* operon, assuming that MerR-like regulators are constitutively synthesized in the bacterial cytoplasm. In the case of ArsR, we cloned both the *parsR* promoter and the *arsR* gene downstream. Indeed, in vivo ArsR regulates its own expression. Then, without the addition to the plasmid of the *arsR* gene under the control of *parsR*, the expression of the bioluminescence would be constitutive.

Carbon source effect on the detection

A carbon source added to the bacterial culture medium was studied in order to evaluate its contribution to the emitted bioluminescence. The inducible *E. coli* DH1 pBznt*lux* strain was grown in four media which differed solely in the origin of the carbon substrate ($C/N/P_{final}$= 100/10/1). Pyruvate, acetate, glucose, and citrate were tested as well as the diluted LB medium, which is mainly used to overcome the auxotrophies. Inductions by cadmium were carried out on bacteria during the stationary growth phase.

Figure 2 shows that the induction ratio depends on the carbon source. The strongest induction ratio was always obtained with the acetate medium (IR=1,700 and 45 for 5 and 0.05 μM of $CdCl_2$, respectively), while growth in the diluted LB provided the weakest signal. This demonstrates that the impact the LB components added to each medium for the purposes of auxotrophies was negligible, and only the carbon source tested (acetate, pyruvate, glucose, and citrate) significantly influenced the induction ratio of the *E. coli* DH1 pBzntlux.

These results highlight the importance of the carbon source for the pre-culture of bacteria before the bioassay in order to optimize the induction ratio. This phenomenon, previously only studied by comparing rich and poor media [16, 40], could influence not only the energy provided to the cells but also the state of the bacterial membranes, which affects the capacity for metal exchange and adsorption [45–47]. Acetate was the carbon source for pre-culture in the remainder of our study.

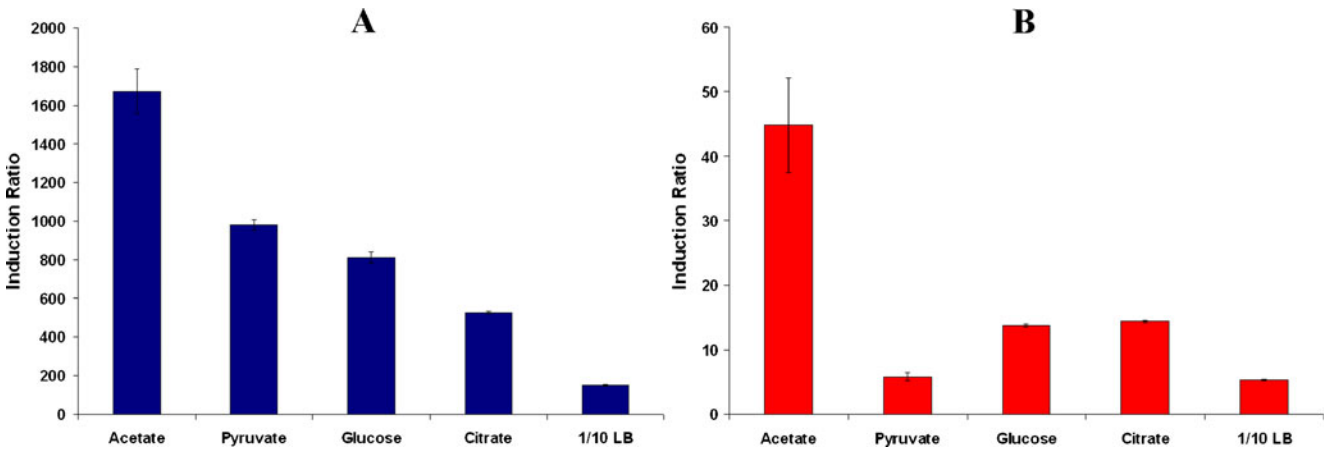


Fig. 2 Effect of the carbon source on the *E. coli* DH1 (pBzntlux) induction ratio performed during the stationary phase of growth and with two cadmium concentrations: (**a**) 5 μM and (**b**) 0.05 μM (N=2 for each medium, and each point is the average of three measurements). Experimental conditions: cells were cultivated for 14 h (30 °C, 250 rpm) in each medium prepared with glucose, acetate, pyruvate, and citrate or only in LB medium diluted ten times. Bacteria in stationary growth phase were then diluted at $OD_{620\ nm}$=0.05 in fresh medium. Inductions were made in microtiter plates. Twenty-five microliters of fivefold concentrated heavy metal solution were added to 100 μL of diluted bacteria. The bioluminescence signal was tracked for 60 min after the addition of the metal, and the results show the maximum induction ratio signal measured

Growth phase effect on bioluminescence

The three strains, *E. coli* DH1 pBzntlux, pBarslux, and pBcoplux, were grown in the acetate medium in batch conditions, and the calculated specific growth rates were very close as expected (0.649, 0.692, and 0.697 h^{-1}, respectively). The induction ratios were extremely reliant on the growth phase and the genetic construction itself (Fig. 3). The induction ratios of the strains *E. coli* DH1 pBzntlux (Fig. 3a) and pBcoplux (Fig. 3c) induced by cadmium and copper, respectively, were at a maximum during the stationary phase (beginning and end of the growth). This phenomenon can be explained by the fact that the ZntR and CueR regulators from the MerR family (specific to heavy metals and considered as stress-sensitive factors) are mainly expressed during the stationary phase of the growth [34, 48]. However, these results differ from Riether et al. [24] and Harkins et al. [49] who reported a maximum of bioluminescence in exponential phase. This disparity could be explained by the use of different induction protocols from bacteria cultivated in a rich and complex LB medium.

In contrast, the induction ratios produced by the strain *E. coli* DH1 pBarslux (Fig. 3b) were recorded in the

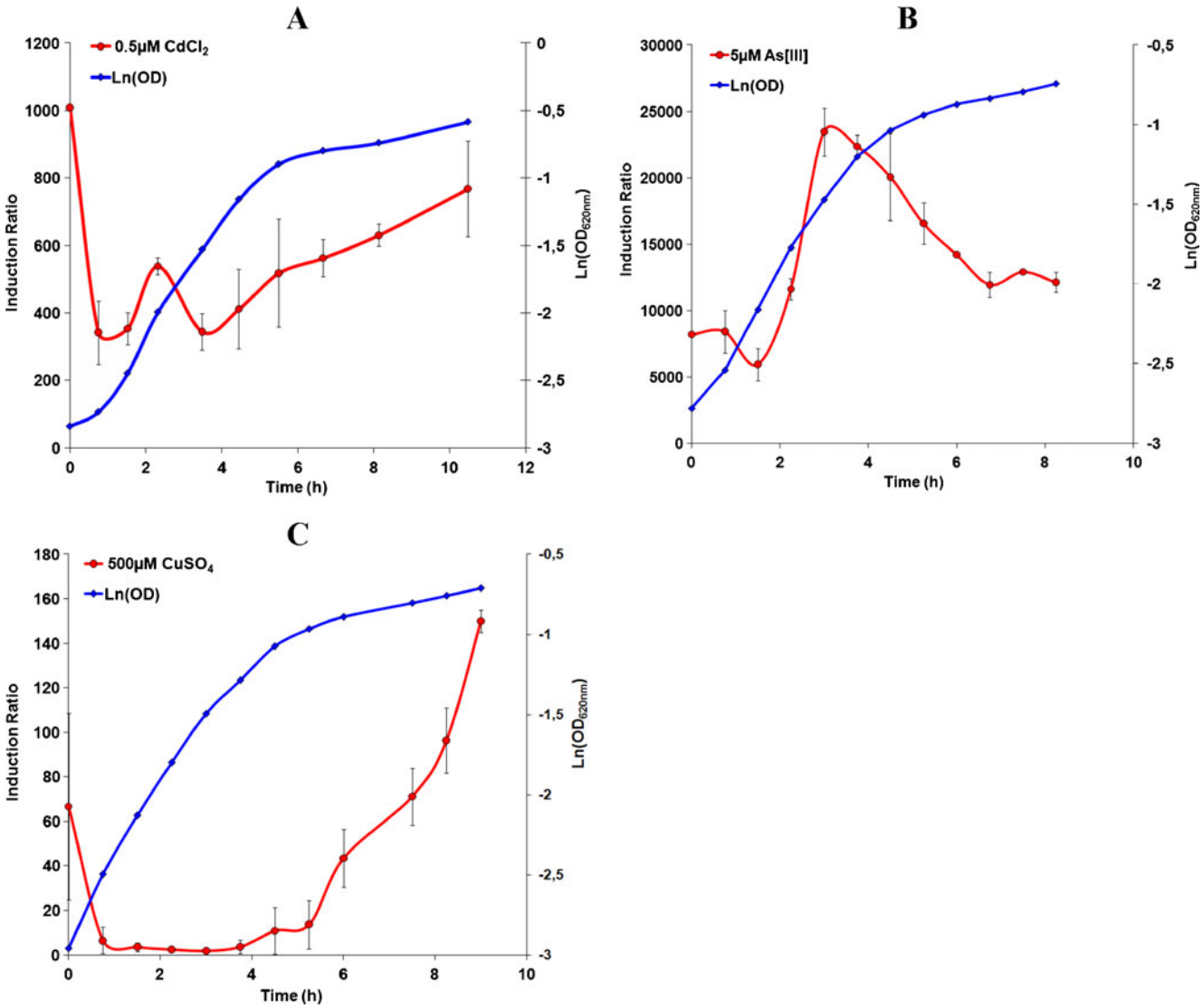


Fig. 3 Growth phases effect on heavy metal detection with *E. coli* DH1 pBzntlux (**a**), pBarslux (**b**), and pBcoplux (**c**; N=2 for each strain, and each point is the average of three measurements). Experimental conditions: cells were cultivated at 30 °C in 1 L Erlen flask containing 200 mL of acetate medium shaken at 250 rpm. Every 1 or 2 h, 2 mL of the culture was taken to measure the $OD_{620\ nm}$ and to monitor bioluminescence after induction with heavy metals as described previously in Fig. 2

exponential phase. Nevertheless, the level of light produced in the stationary phase was five to six times higher than for *E. coli* DH1 pBzntlux, allowing the possibility of using the strain *E. coli* DH1 pBarslux in this phase. This difference could be attributed to (1) the genetic construction from the SmtB/ArsR family [37] and (2) the regulator that is synthesized from the plasmid and not only from the chromosome, as it is the case for the two other strains.

Sensitivity and specificity of the bioelements

In order to determine the possible cross-detection between the three sensitive strains, 15 heavy metals were selected and tested either for their considerable toxicity or for their abundance in the environment (Table 3).

From the set of tested metals, 12 induced at least one of the three strains. Cadmium and mercury were detected by all the strains with a detection limit of 5 nM. Taurianen et al. [23] reported a detection limit of 10 nM for cadmium with a different genetic construction in *Staphylococcus aureus*. Lead, tin, and arsenic III and V were detected by *E. coli* pBzntlux and pBarslux. The detection limit (5 nM) for As_2O_3 with the *E. coli* pBarslux was very low, while the strain was induced by six metals in comparison with three metals (As, Sb, and Cd) as reported by Hakkila et al. [38]. Five metals, zinc, nickel, cobalt, chromium VI, and copper, were only detected by one strain. Lastly, chromium III, iron II, and manganese were not detected. Cadmium, lead, tin, and arsenic V induced a common response for two or three bioluminescent strains.

As previously pointed out [24, 50, 51], the bioluminescent bacteria were not specific to only one metal but rather to a range of metals. This drawback could be overcome if bacteria were to be used in a battery of responses, thereby taking advantage of the cross-detections in the biosensor to narrow the range of detected metals as suggested by the close-up which has been added to Table 3. For example, when *E. coli* DH1 pBzntlux cells are induced with an unknown liquid sample, according to Table 3, the possible metals present are Cd, Hg, Pb, Sn, As, Zn, Ni, Co, and Cr. If the three strains are induced at the same time, the resulting bioluminescence is only induced with Cd or Hg that entail a cross-reaction for the three reporter bacteria.

Choice of the immobilization procedure and effect on the detection

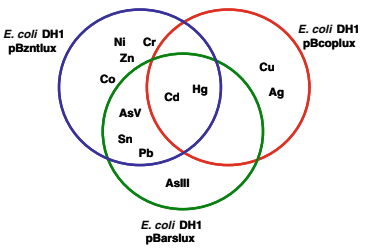
Three methods can be used to immobilize microorganisms in a biosensor: (1) covalent binding on a support, (2) physical adsorption on a membrane-like cellulose (hydrogen bonds, Van der Walls links, etc.), and (3) encapsulation or inclusion of the microorganisms in a reticulated neutral and biocompatible aqueous matrix. Above all, this latest method remains simple and economic [52, 53].

Bacteria in our biosensor were immobilized in two biocompatible matrix types for comparison purposes: synthetic PVA and agarose as a biological material. Alginate and κ-Carrageenan were rejected due to their strong metal absorption capacities [54].

As depicted in Fig. 4a, the peak for induction ratios after induction of *E. coli* DH1 pBzntlux strains was strongly influenced by the immobilization matrix, agarose having a clear advantage (41 min for suspended bacteria, 67 min for bacteria in agarose, and 117 min for bacteria in PVA). As well as a delay for the kinetic detection, as pointed out by Affi et al. [55], the second effect was a decrease in the induction ratios, with a drop of 99% for PVA and 43% for agarose compared to induction ratios for the suspended

Table 3 Heavy metals detected by the strains *E. coli* DH1 pBzntlux, pBarslux, and pBcoplux

Heavy metal tested	Detection limit (μM)			ES (μM)[a]
	E. coli DH1 pBzntlux	*E. coli DH1* pBcoplux	*E. coli* DH1 pBarslux	
$CdCl_2$	0.005	0.05	0.5	0.045
$HgCl_2$	0.005	5	0.5	0.005
$PbCH_3CH_2O$	5	nd	50	0.005
$SnCl_2$	5	nd	500	/
As_2O_5	50	nd	0.05	0.135
As_2O_3	nd	nd	0.005	0.135
$ZnCl_2$	5	nd	nd	nc
$NiSO_4$	50	nd	nd	0.34
$CoCl_2$	50	nd	nd	nc
$K_2Cr_2O_7$	50	nd	nd	1
$CrCl_3$	nd	nd	nd	1
AgNo3	nd	0.5	nd	/
$CuSO_4$	nd	50	nd	30
$FeSO_4$	nd	nd	nd	nc
$MnCl_2$	nd	nd	nd	/



Cross reaction of the three strains

ES European standards in water, *nd* metal tested but not detected by the strains, / unavailable data, *nc* not concerned by a standard

[a] Obtained from INERIS (Institut National de l'Environnement Industriel et des Risques, France)

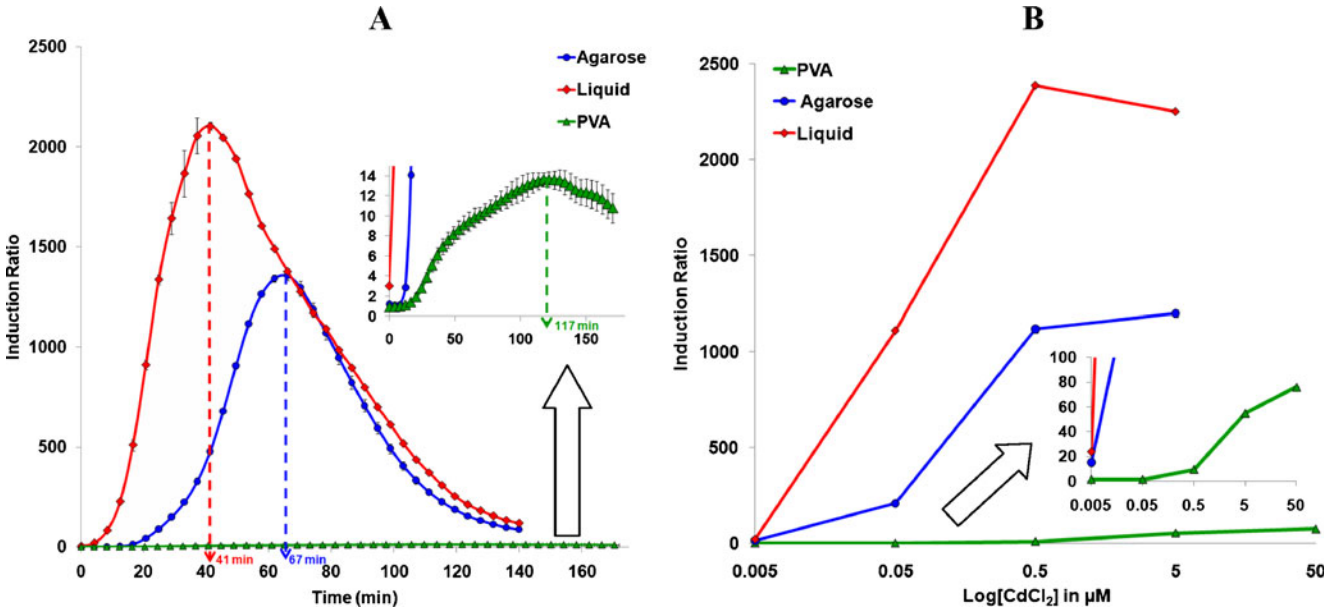


Fig. 4 Comparison of bioluminescence inductions with cadmium of *E. coli* DH1 pBzntlux in liquid phase and immobilized either in agarose (2%) or in PVA (10%). **a** Induction ratios measured over time after the addition of cadmium (0.5 µM $CdCl_2$) until the maximum signal. **b** Cadmium detection range of *E. coli* DH1 pBzntlux in liquid phase or immobilized either in agarose or PVA. The *insets* are a close view of the low induction ratio produced by the bacteria immobilized in the PVA matrix (N=2 for each condition, and each point is the average of three measurements. The standard error was added but is sometimes very low and hidden by the *symbols*). Experimental conditions: 100 µL of bacteria immobilized either in agarose 2% or in PVA (10%) or in liquid phase was introduced in microtiter plates at $OD_{620\ nm}$≈0.1. Bioluminescence inductions were achieved with the addition of 25 µL of fivefold concentrated $CdCl_2$ solution. The bioluminescence signal was tracked for 2 h after the addition of the metal

bacteria. The third effect from the use of PVA was a decrease in the detection limit for $CdCl_2$ that was not measured for agarose (DL_{liquid}=0.005 µM, DL_{PVA}=0.05 µM, $DL_{agarose}$= 0.005 µM, Fig. 4b). The same trends were assessed for *E. coli* DH1 pBarslux and *E. coli* DH1 pBcoplux immobilized in agarose (Fig. 5).

Immobilization system must be accurately selected in order to ensure survival of active cells, signal detection,

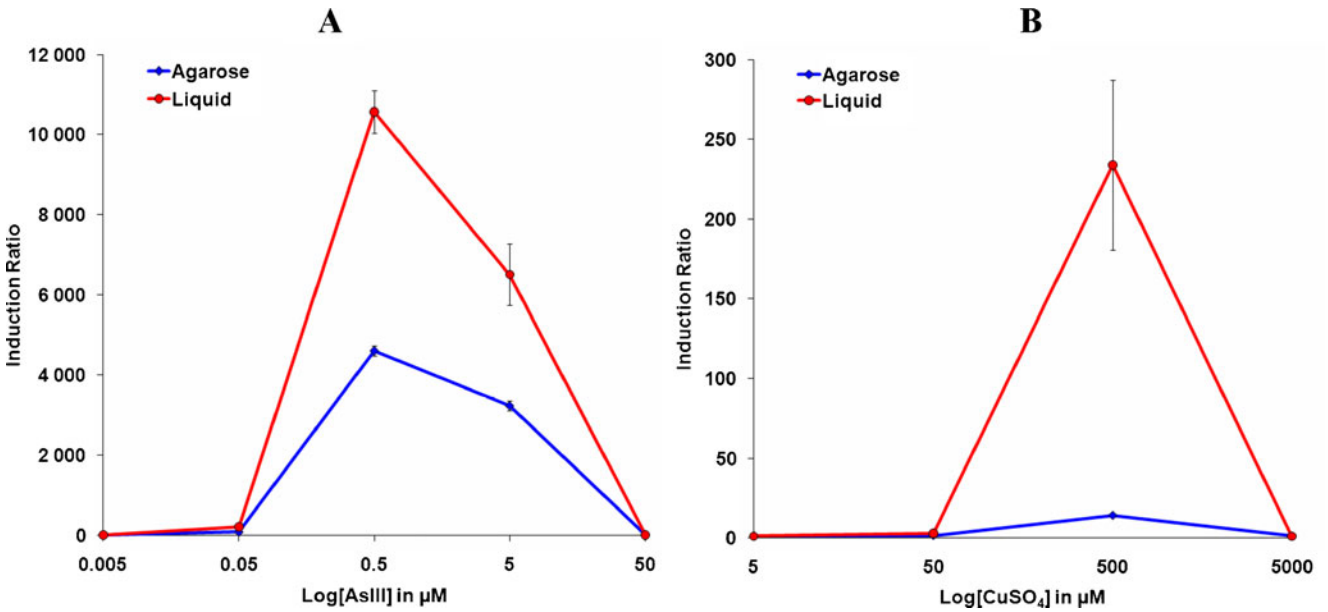


Fig. 5 Agarose matrix effect (2%) compared to liquid phase in bioassay on arsenic (III) and copper (II) detection range, respectively, by *E. coli* pBarslux (**a**) and *E. coli* pBcoplux (**b**; N=2 for each strain in each immobilization condition, each point is the average of three measurements). Experimental conditions were as described in Fig. 4

easy and rapid measurement, and long-term storage [56].

Agarose is a linear disaccharide polymer that shows a high biocompatibility, transparency, and good stability properties [57]. Agarose is promising, as its purity guarantees a transparent gel and lower melting temperatures. Agarose has good diffusion properties, and heavy metals are generally not bound in agarose network. Studies on microbial biosensor for heavy metal detection showed that bacteria immobilized in agarose emitted light signal twofold higher that the ones in alginate [58], probably due to metal adsorption in the latter. Some other studies employed pure low melting agarose to immobilize luminescent bacteria for pollutant detection [59–62].

PVA compared to agarose in this study is a white soluble high-molecular compound, not soluble in ordinary organic solvents. It shows excellent tensile stress, mouldability, impact strength, wear resistance, and excellent electrical insulation. Generally used as raw material for coating and adhesives, PVA shows interesting features for cell immobilization [63]. PVA containing bacteria may gel through different procedures. We used the very simple freeze-thawing procedure, and it proved efficient for bacteria immobilization for organo-phosphorus neurotoxins detection [64] and toxicity assessment in wastewater [65]. Nevertheless, a modified PVA, which allows immobilization in lens-shaped discs, was applied to BOD biosensors [66], but a significant reduction in signal emission was observed. In our study, the bioluminescence signal and the sensitivity were both altered due to a probable effect of the freezing process of PVA polymerization. Conversely, agarose offered a good compromise in terms of simplicity, harmlessness of the procedure, and transparency. This matrix was used in the biosensor as described in part 2.

Conclusion

Initially, three strains were used in order to detect a broad range of heavy metals. Their sensitivities were evaluated and as a consequence of the cross-reaction of the three strains towards metals, it is anticipated that a better discrimination among metals will be possible (for example, Cd and Hg). Nevertheless, our current approach does not address the possible antagonistic or synergistic effects of metals in a mixture.

In order to use these bacteria in a biosensor, their characterization was carefully completed and took into consideration (1) the carbon source that dramatically influences the induction ratios (acetate was chosen) and (2) the growth phase effect that depends on the strains. Induction ratios were significantly better on stationary phases for both *E. coli* pBzntlux and *E. coli* pBcoplux, while the exponential phase was suitable for *E. coli* pBarslux. Finally, PVA and agarose were compared for their effect on the detection time, the detection limit, and the induction ratios output. We demonstrated that agarose was the best matrix.

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