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Luminescent bacterial sensor for cadmium and lead

Sisko Tauriainen, Matti Karp, Wei Chang, Marko Virta *

University of Turku, Department of Biotechnology, Tykistökatu 6, FIN-20520, Turku, Finland

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Abstract

A sensor plasmid was constructed by inserting the regulation unit from the *cadA* determinant of plasmid pI258 to control the expression of firefly luciferase. The resulting sensor plasmid pTOO24 is capable of replicating in Gram-positive and Gram-negative bacteria. The expression of the reporter gene as a function of added extracellular heavy metals was studied in *Staphylococcus aureus* strain RN4220 and *Bacillus subtilis* strain BR151. Strain RN4220(pTOO24) mainly responded to cadmium, lead and antimony, the lowest detectable concentrations being 10 nM, 33 nM and 1 nM respectively. Strain BR151(pTOO24) responded to cadmium, antimony, zinc and tin at concentrations starting from 3.3 nM, 33 nM, 1 μM and 100 μM, respectively. The luminescence ratios between induced and uninduced cells, the induction coefficients, of strains RN4220(pTOO24) and BR151(pTOO24) were 23–50 and about 5, respectively. These results were obtained with only 2–3 h incubation times. Freeze-drying of the sensor strains had only moderate effects on the performance with respect to sensitivity or induction coefficients.

Keywords: Luciferase; Genetically modified microorganism; Metal bioavailability; Bioluminescence; Cadmium; Lead

1. Introduction

Some heavy metals, such as nickel, cobalt and zinc, are essential to micro-organisms as trace nutrients, in contrast to others such as cadmium and lead, which have no known beneficial roles (Nies, 1992). However, all heavy metals are toxic in micro- or millimolar concentrations. Yet certain bacteria are capable of growing in metal contaminated areas. These bacteria are usually adapted to the presence of toxic metal by genetically encoded resistance mechanisms, the expression of which is precisely regulated. The resistance mechanism towards many toxic metals, including cadmium, works by the exclusion of metals by an energy-dependent pump in the cell membrane. The precise regulation of the resistance gene expression has been utilized in the construction of specific sensor bacteria, in which the regulatory element is connected to control the expression of a reporter gene. Operons that have been utilized include, for example, the *mer* operon (Selifonova et al., 1993; Tescione and Belfort, 1993; Virta et al., 1995), which codes for the resistance towards mercury, the *ars* operon (Corbisier et al., 1993; Scott et al., 1997; Tauriainen et al., 1997), which codes for the resistance to arsenic and antimony and the *cadA* operon (Corbisier et al., 1993), which codes for the resistance to cadmium, zinc (Novick and Roth, 1968; Yoon and Silver, 1991) and possibly also for the resistance to lead and bismuth (Novick et al., 1979; Smith and Novick, 1972). The cadmium resistance operon from *Staphylococcus aureus* plasmid pI258, *cadA*, consists of two genes: the *cad*A and the *cad*C (Nucifora et al., 1989; Yoon and Silver, 1991). The *cad*C gene encodes for the regulatory protein (Endo and Silver, 1995) and the *cad*A gene for an energy-dependent ion pump (Nucifora et al., 1989), which is responsible for the cadmium efflux from the cells.

Luciferases are a group of heterogeneous enzymes with the ability to produce light as a byproduct of their catalyzed reactions. The firefly luciferase (*luc*FF) is widely used as a reporter gene in prokaryotic as well as in eukaryotic systems because it can provide sensitive and simple detection of gene expression and regulation. The quantification of light emission, i.e. bioluminescence, is one of the most sensitive means of detection and can be measured with a liquid scintillation counter, a luminometer or even with X-ray film.

We describe here the construction of a recombinant bacterial strain for measuring bioavailable cadmium and

* Corresponding author. Phone: + 358-2-3338085; Fax: + 358-2-3338050; E-mail: marvirta@utu.fi

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lead. Sensor plasmid pTOO24 carries the firefly luciferase reporter gene under the control of the *cad* promoter and the *cad*C gene of the *cadA* resistance determinant of *S. aureus* plasmid pI258 (Nucifora et al., 1989; Tynecka et al., 1981). As host strains we used *S. aureus* RN4220 and *Bacillus subtilis* BR151.

2. Materials and methods

2.1. Materials

Tryptone, Yeast Extract and hydrolyzed casein were obtained from Difco. β-glycerophosphate was from Sigma. D-luciferin was from Bio-Orbit Oy (Turku, Finland). $NaAsO_2$, Na_2HAsO_4, $FeSO_4$, Li_2SO_4 were from Sigma, $HgCl_2$, $CdCl_2$, $CuSO_4$, $CoCl_2$, $MnCl_2$ were from Riedel-de Haën, $ZnCl_2$, $SnCl_2$, $NiCl_2$, were from Merck, $C_4H_4KO_7Sb$ was from Fluka and $Pb(CH_3COO)_2$ was from J.T. Baker. All metals were of analytical reagent grade, except $C_4H_4KO_7Sb$, which was of purum grade ($\geq$ 99%). DNA modifying enzymes were obtained either from Promega or from New England Biolabs. Vent DNA-polymerase used in PCR was from New England Biolabs.

2.2. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. Plasmid containing bacterial strains were maintained on L-agar (LA) plates supplemented with 30 μg/ml kanamycin.

2.3. Construction of plasmid pTOO24

The original *lac*-operator/T5-promoter element of pCSS810 was replaced by the cadmium responsive regulation unit (Fig. 1). The inserted unit contained the *cad*C gene, which codes for the regulatory protein (Endo and Silver, 1995), and the *cad* promoter upstream of the *luc*FF gene. Plasmid pTOO24 was constructed on a similar principle to the previous sensor plasmids described by us (Tauriainen et al., 1997; Virta et al., 1995) by using standard recombinant-DNA techniques (Sambrook et al., 1989). Plasmid pI258 isolated from *S. aureus* (NCTC 50581) was used as a template for polymerase chain reaction (PCR) (Saiki et al., 1985) to generate a fragment consisting of the *cad*C gene and promoter/operator of the *cadA* operon with *Bam*HI and *Xho*I restriction sites at ends. Following oligonucleotide primers were used for PCR: 5′-ATAT**CTCGAG***GTGT ATTTTTTAATAAATTATTTTACTT*-3′ at the beginning of the *cad*C gene and 5′-TTAA**GGATCC**CCTT*TCAGA CATTGACCTTCAC*-3′ at the end of the *cad*C gene, showing restriction sites of *Xho*I and *Bam*HI in bold, respectively, and bases which correspond to the *cadC* gene in italics. The 5′ end of the generated fragment included seven bases from the beginning of the *luc*FF because the *Bam*HI site was originally engineered inside the luciferase gene (Lampinen et al., 1992). The resulting PCR product, the 572 bp fragment, was purified by using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany), digested with *Bam*HI and *Xho*I and repurified from an agarose gel by QIAquick gel extraction kit (Qiagen GmbH). The fragment was ligated to plasmid pCSS810 (Lampinen et al., 1992), from which the constitutive promoter controlling *luc*FF expression was removed with *Bam*HI and *Xho*I digestion and gel purification. The generated plasmid pTOO24 was transformed into *Escherichia coli* strain MC1061 cells by electroporation (Dower et al., 1988), isolated and its structure confirmed by restriction enzyme digestion and sequencing. Plasmid pTOO24 was also transformed by electroporation into *S. aureus* (Schenk and Lad-

Table 1
Bacterial strains and plasmids used

Strain or plasmid	Description	Reference/source
Strain:		
Escherichia coli		
MC1061	*c*I$^+$ Δ(*ara*,*leu*)7697 Δ*lac*X74 *gal*U$^-$ *gal*K$^-$ *hsr*$^{--}$ *hsm*$^+$ *rps*L *ara*D139	(Casabadan and Cohen, 1978)
Bacillus subtilis		
BR151	*lys*-3 *met*B10 *trp*C2	(Young et al., 1969)
Staphylococcus aureus		
NCTC50581	Multiple metal resistant pI258	(Novick et al., 1979)
RN4220	Efficient acceptor of *E. coli* DNA	(Kreiswirth et al., 1983)
Plasmid:		
pCSS810	Shuttle vector, T5 promoter—*lac* operator upstream of *luc*FF gene, kanamycin and chloramphenicol resistances	(Lampinen et al., 1992)
pTOO24	Shuttle vector, *cad* promoter and *cad*C of pI258 cloned into pCSS810 upstream of *luc*FF gene, kanamycin and chloramphenicol resistances	This work

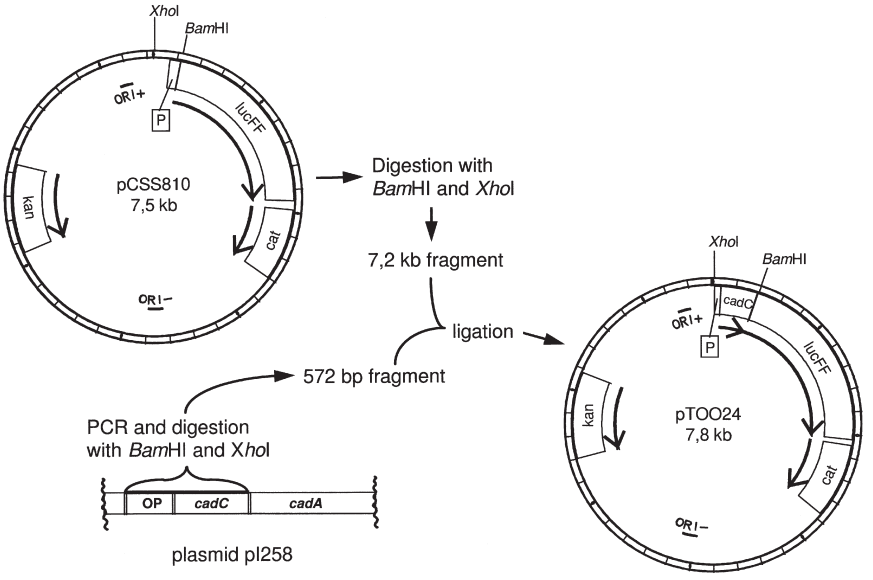

Fig. 1. Construction of the sensor plasmid pTOO24. Plasmid pCSS810 was digested with B*am*HI and *Xho*I and the resulting 7,2 kb fragment was isolated. A 572 bp fragment was amplified from plasmid pI258 by PCR with the primers described in Section 2. The 572 bp fragment was purified, cut with *Bam*HI and *Xho*I, repurified and ligated to the 7,2 kb fragment from pCSS810. Abbreviations used: *kan*, gene encoding kanamycin resistance; *cat*, gene encoding chloramphenicol resistance; *cadC*, gene encoding the regulative protein of the *cad* promoter; P, the *cad* promoter; *luc*FF, gene encoding firefly luciferase; *ori* + and *ori*-, origins of replication in Gram + and Gram − bacteria, respectively.

daga, 1992) strain RN4220 and *B. subtilis* (Vehmaanperä, 1990) strain BR151. *S. aureus* RN4220 harbouring the parental plasmid pCSS810 was used as a control strain since in pCSS810 the expression of the *luc*FF is controlled by *lac* operator and thus independent of metal concentration.

2.4. Cultivation of bacteria

Bacterial cultivations for the measurements were done in LB-medium (10 g Tryptone, 5 g Yeast extract, 5 g NaCl per litre, pH 7.0) supplemented with 30 μg/ml kanamycin in a shaker at 30°C. The *S. aureus* cells were grown to an OD_{600} nm of 4–6 and *B. subtilis* to an OD_{600} nm of 0.2. Cells were harvested by centrifugation and washed twice: *S. aureus* with HMM-medium (LaRossa et al., 1995) supplemented with 0.25% hydrolyzed casein, and *B. subtilis* with modified SMM-medium (2 g $(NH_4)_2SO_4$, 14 g K_2HPO_4, 6.8 g $NaH_2PO_4 \cdot 2H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$, 50 ml 10% glucose solution per liter, pH 7.3), supplemented with 0.05% hydrolyzed casein. Bacteria were suspended in and diluted with the same media before the measurements. About 1×10^6 *S. aureus* cells and 5×10^5 *B. subtilis* cells were used per measurement.

2.5. Luminescence measurements

The bacterial dilution and different dilutions of metal salt solutions were pipetted to wells of 96-well white microtitration plates (Labsystems, Helsinki, Finland), both in a volume of 50 μl. Plates were incubated at 30°C without shaking for 120 min with *S. aureus* cells and for 180 min with *B. subtilis* cells. The *in vivo* luminescence peak values were measured with a Luminoskan luminometer (Labsystems, Helsinki, Finland), by adding 100 μl of the luciferase substrate (1 mM D-luciferin in 0.1 M Na-citrate buffer, pH 5.0) through the dispenser of the luminometer. All measurements were done in triplicate. The metal salt solutions used were made in Milli-Q - purified (Millipore, Bedford, Massachusetts) water. These solutions were: $CdCl_2$, $Pb(CH_3COO)_2$, $C_4H_4KO_7Sb$, $SnCl_2$, $ZnCl_2$, $HgCl_2$, $MnCl_2$, Li_2SO_4, $NaAsO_2$, Na_2HAsO_4, $FeSO_4$, $CuSO_4$, $NiCl_2$ and $CoCl_2$.

Induction coefficients were calculated in order to determine the efficiency of induction with different metals. The induction coefficient was calculated as the ratio between induced and uninduced cells as follows: Induction coefficient $I = I_I/I_B$, where I_I is the light emitted by the induced sample and I_B is the light emitted by the uninduced sample, the background light.

Survival of control strain RN4220(pCSS810) is the ratio of luminescence of the zero sample and metal containing samples multiplied by a factor of 100. It was calculated as follows: survival of control strain $S = S_s/S_Z \times 100\%$, where S_s is the light emitted by the cells incubated with a metal containing sample and S_Z is the light emitted by the cells incubated with the zero sample.

2.6. Freeze-drying of cells

Both the *S. aureus* strain RN4220(pTOO24) and the *B. subtilis* strain BR151(pTOO24) were preserved by freeze-drying. An overnight culture of the cells was

diluted 1:50 into 50 ml of LB-medium supplemented with 30 μg/ml kanamycin. Cells were cultivated at 30°C in a shaker, *S. aureus* to an OD_{600} nm of 3 and *B. subtilis* to an OD_{600} of 0.2, after which they were harvested by centrifugation. Cells were suspended into 50 ml of medium, M9-medium (Sambrook et al., 1989) for *S. aureus* and modified SMM-medium for *B. subtilis*, both media supplemented with 10% of lactose. Freeze-drying was performed according to standard procedures (Janda and Opekarova, 1989; Sidyakina and Golimbet, 1991) in 200 μl aliquots using a Lyoflex 10 freeze-dryer (Edwards Inc, Crawley, UK). Cells were reconstituted by adding 200 μl of distilled water and luminescence measurements were done as with fresh cells.

3. Results

3.1. Regulation of luciferase expression and optimization of reaction conditions

In the absence of inducing metals like cadmium, the expression of the luciferase gene was low. In addition, the chloramphenicol acetyltransferase (*cat*) gene located downstream from the luciferase gene, seemed to be repressed because *S. aureus* RN4220(pTOO24) cells did not grow in the presence of 30 μg/ml chloramphenicol. The parental plasmid containing *S. aureus* strain, RN4220(pCSS810), in which luciferase and chloramphenicol acetyltransferase are constitutively expressed, grew in those conditions (data not shown).

Incubation time, medium composition, growth phase of harvested bacteria and amount of bacteria per measurement were optimized. Also inductions in different host strains were tested and optimization of conditions was done for *B. subtilis* strain BR151 in addition to *S. aureus* strain RN4220. Medium composition and incubation time with metals had an effect on the induction profiles of both strains. *S. aureus* cells were tested in following media: LB, M9 (Sambrook et al., 1989) and HMM (LaRossa et al., 1995) (Fig. 2). M9 and HMM were both supplemented with 0.25% Casamino acids. Additionally, *B. subtilis* was tested in SMM (Harwood and Cutting, 1990) and modified SMM, which were both supplemented with 0.05% Casamino acids. The minimal medium containing glycerophosphate instead of phosphate, HMM, proved to be the most sensitive for both cadmium and lead detection with *S. aureus* RN4220(pTOO24) and it was therefore used in further measurements. In addition, the induction coefficient caused by lead was higher compared with the phosphate containing minimal medium (M9). Modified SMM was chosen for *B. subtilis* BR151(pTOO24) because of the improvements in sensitivity and induction coefficients in cadmium detection. Incubation time for maximal induction was two hours for RN4220(pTOO24) and three hours for BR151(pTOO24) (data not shown). In addition to media, the growth phase of harvested cells affected the induction of BR151(pTOO24), early stages of growth (OD_{600} of 0.2) gave the best results. Other alterations had no effect on the luminescence curves.

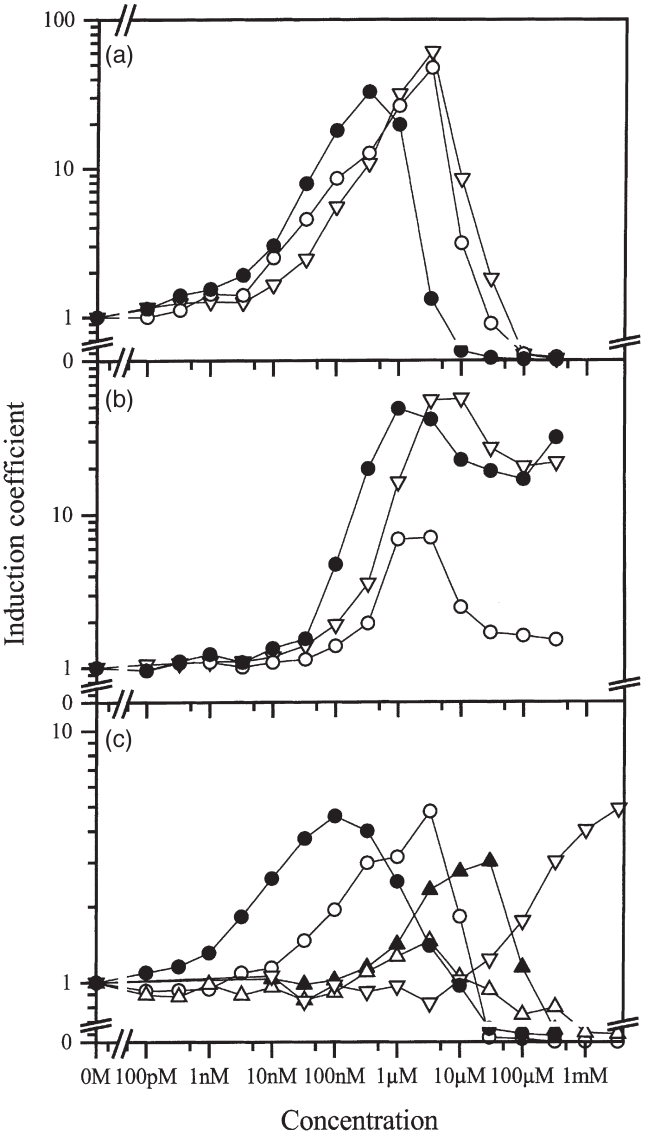

Fig. 2. The effect of medium composition on induction of *S. aureus* RN4220(pTOO24) and induction profiles of *B. subtilis* BR151(pTOO24). (a) and (b) show the induction coefficients of strain RN4220(pTOO24) when incubated at 30°C for 2 h with (A) cadmium and (B) lead in different media: LB (▽), M9 supplemented with 0.25% hydrolyzed casein (○) and HMM supplemented with 0.25% hydrolyzed casein (●). (c) shows induction profiles of strain BR151(pTOO24) when incubated at 30°C for 3 h with cadmium (●), antimony (○), zinc (▲), tin (▽) and lead (△). Luminescence measurements and calculation of the induction coefficients are described in Section 2.

3.2. Induction of S. aureus *RN4220(pTOO24) with different compounds*

The following ions caused induction of luminescence: cadmium, lead, antimonite and tin (Fig. 3). Minor levels

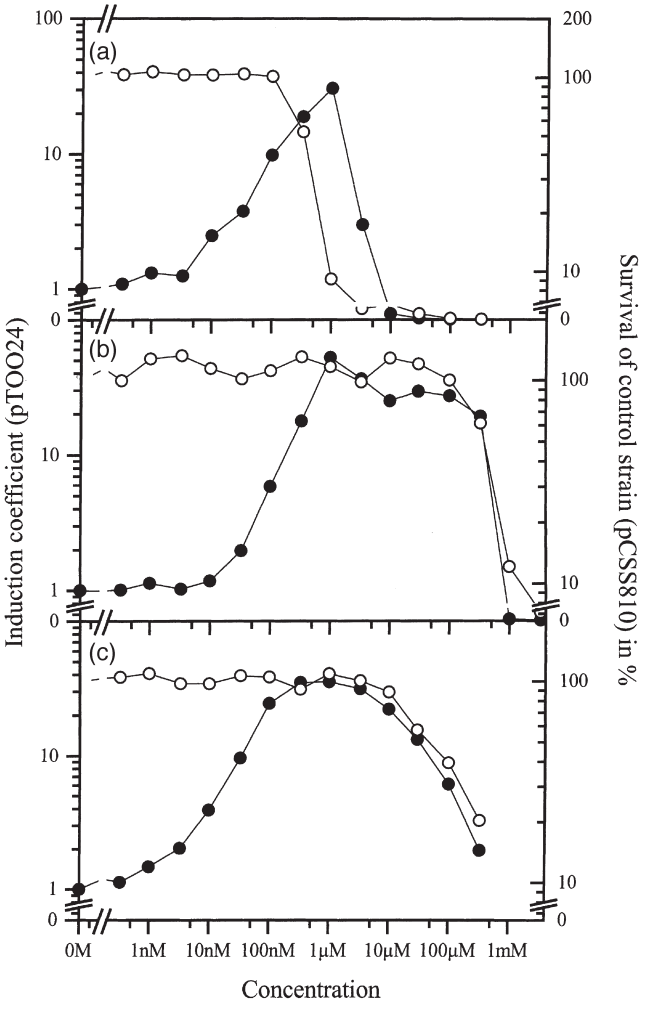

Fig. 3. Response of *S. aureus* strains to cadmium, lead and antimony. Induction coefficients of the cadmium and lead-sensing strain *S. aureus* RN4220(pTOO24) (●) and the survival of the stable-light producing control strain *S. aureus* RN4220(pCSS810) (○) when incubated with (a) cadmium, (b) lead and (c) antimony at 30°C for 2 h are shown. Data represent mean of three determinations. Luminescence measurements and calculation of the induction coefficients and the survival of the control strain are described in Section 2.

of luminescence were induced also by zinc, mercury and manganese. Cadmium, lead and antimony all induced rather different shaped curves. The lowest concentration that caused a noticeable induction (background + 2 × standard deviation) with cadmium (Cd^{2+}) was 10 nM. Luminescence increased with increasing amounts of Cd^{2+} ions in a quite linear manner to a concentration of 1 μM, after which luminescence fell and reached the background value at 10 μM. Concentrations needed for luminescence with lead (Pb^{2+}) were from 33 nM to 330 μM. Pb^{2+} ions induced a somewhat different luminescence response: luminescence rose linearly between concentrations from 33 nM to 1 μM and then fell at 330 μM to 1 mM. At concentrations from 1 μM to 330 μM the luminescence was at a high level. Antimonite (SbO_2^-) was also an effective inducer of light emission. SbO_2^- ions induced luminescence between concentrations of 1 nM to 330 μM, resulting in a bell-shaped curve, which had its maximum induction capacity at concentrations of 330 nM- − 1 μM. Tin (Sn^{2+}) induced luminescence at concentrations from 3.3 μM to 100 μM; the curve had its peak at 33 μM. The maximum induction coefficients for the different metals were 30, 50, 35, and 23 for cadmium, lead, antimony and tin, respectively. $HgCl_2$, $ZnCl_2$ and $MnCl_2$ induced less luciferase synthesis at narrow concentration ranges: $HgCl_2$ from 33 nM to 100 nM, $ZnCl_2$ from 1 μM to 10 μM and $MnCl_2$ from 33 μM to 330 μM with maximum induction coefficients of 3–6. The following compounds, $FeSO_4$, Li_2SO_4, $CuSO_4$, $NaAsO_2$, Na_2HAsO_4, $NiCl_2$, and $CoCl_2$ did not cause significant bioluminescence even at millimolar concentrations (data not shown).

3.3. Induction of B. subtilis BR151(pTOO24) with different compounds

Metals which induced luminescence in BR151(pTOO24) were cadmium, antimonite, zinc and tin (Fig. 2). Ion concentrations needed were from 3.3 nM to 1 μM, 33 nM to 10 μM, 1 μM to 33 μM and starting at 100 μM, respectively, with induction coefficients of 3–5.

3.4. The effect of metals on the control strain S. aureus RN4220(pCSS810) producing constitutive light

Luminescence was at a constant high level at low concentrations of all metals and the fall in luminescence occurred at various concentrations due to differences in metal toxicity. Cd^{2+} ions caused luminescence to fall at a concentration of 100 nM (Fig. 3). It reached the background value at a concentration of 33 μM. The fall in luminescence caused by Pb^{2+} ions occurred at concentrations from 100 μM to 3.3 mM and that of Sn^{2+} ions at concentrations from 10 μM to 3.3 mM. With SbO_2^- ions the reduction of light starts at a concentration of 10 μM but does not reach the background value in concentrations tested.

3.5. The effect of freeze-drying on cells

Freeze-drying of *S. aureus* RN4220(pTOO24) cells resulted in lowered induction coefficients and somewhat reduced sensitivity of both cadmium and lead detection (Fig. 4). Freeze-drying had a smaller effect on *B. subtilis* BR151(pTOO24) cells, only the sensitivity decreased slightly (data not shown).

4. Discussion

The recombinant sensor bacteria, *S. aureus* RN4220(pTOO24), presented here responds mainly to

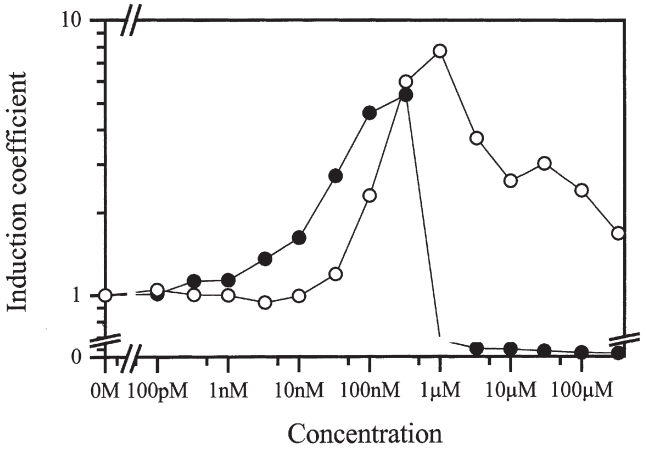

Fig. 4. Induction of freeze-dried RN4220(pTOO24) cells. Induction coefficients of freeze-dried RN4220(pTOO24) cells when incubated at 30°C for 2 h with cadmium (●) and lead (○). Luminescence measurements and calculation of the induction coefficients are described in Section 2.

cadmium, lead and antimony (Fig. 3). These metals are strong inducers and they cause induction of luminescence at a wide concentration range. Tin also causes relatively strong induction, but only over a narrow concentration range and at higher metal concentrations. It is obvious that the shape of concentration–response curve is defined, in addition to the induction of the *cadA* promoter, by the toxicity of a metal. That was confirmed using a control strain, *S. aureus* RN4220(pCSS810), for toxicity measurement. It showed that the fall in luminescence (Fig. 3) is due to the toxic effect of the metal, which is paralleled by reduction of the number of living bacteria. A direct correlation between viable microbial cell count and *in vivo* firefly luciferase activity has been shown previously (Virta et al., 1994). By using the control strain together with the metal-specific strain it should be possible to distinguish whether the metal concentration of samples is on the increasing or on the decreasing part of the concentration–response curve (Fig. 3).

The regulation of the *cadA* resistance determinant has been studied earlier by Yoon et al. (1991) and Corbisier et al. (1993). Yoon *et al.* used a *cad*A-*bla*Z fusion in *S. aureus* RN4220 and reported strong induction with cadmium, lead and bismuth and minor induction with zinc and cobalt. Corbisier *et al.* found cadmium to be the strongest inducer in *S. aureus* RN4220 together with lead and bismuth as minor inducers by using a *cad*A-*lux*AB fusion. Our results are in agreement with these reports with the exception that cobalt did not cause induction in our experiments. We discovered, in addition, that antimonite is an inducer of the *cad* promoter, which has not been reported earlier. Yoon *et al.* and Corbisier *et al.* did not test the inducibility of their strains with antimonite. The induction of a cation-resistance operon by antimonite, which is an oxyanion, seems rather unexpected. On the other hand the arsenite and antimonite resistance operon (*ars*) from the same plasmid pI258 has shown to be inducible also by Cd^{2+} and BiO^{+} (Corbisier et al., 1993; Tauriainen et al., 1997). Furthermore, it has been noticed that the assumed metal binding regions of all proteins of the ArsR family are conserved and the regulation protein of the *cad*A-operon, CadC, is considered a member of the ArsR family like the ArsR protein of the *ars*-operon (Shi et al., 1994; Silver et al., 1993). Interestingly, arsenite did not induce luminescence of strain RN4220(pTOO24), whereas cadmium has been found to induce luminescence of strain RN4220(pTOO21) (Tauriainen et al., 1997), in which the expression of luciferase was regulated by the *ars*-promoter and ArsR-protein. It is clear that antimonite was a common inducer of both strains.

The sensitivity of heavy metal ion detection obtained here exceeded the sensitivities of the above mentioned strains described by Corbisier *et al.* and Yoon *et al.*, which can be considered for use in the detection of cadmium and lead, although they were constructed in order to study the regulation of the *cadA* operon. The detection limits on those studies were about 0.5 μM or higher for cadmium and 1 μM or higher for lead, which are almost one hundredfold greater than the concentrations obtained here. We have obtained similar results with the other sensor bacteria, although the differences compared with sensor bacteria described by others have varied from slightly less than a hundredfold (Tauriainen et al., 1997) up to about millionfold (Virta et al., 1995). The explanation of the differences in sensitivity is not totally obvious to us. One explanation could be the difference in the sensitivity of detection between firefly luciferase and bacterial luciferase. Also the variations in the level of background activity of the reporter and the intracellular metabolism of strains used can affect the sensitivity. Rasmussen et al. (1997) were able to increase the sensitivity of the mercury sensor bacteria by several orders of magnitude simply by using lower amounts of cells per measurement. However, we did not observe similar dependence between cell number and sensitivity in our case. Also the differences in the copy number of the sensor plasmids might play a role in sensitivity of ion detection in formulating the interrelationships between repression and induction.

Of the other strains tested for the sensor plasmid pTOO24, *S. aureus* strain RN4220 was found to be generally the best host, i.e. the most sensitive and the one with the highest induction coefficients for different metal ions. On the other hand *B. subtilis* strain BR151 was the most sensitive for cadmium detection, although it had lower induction coefficients with all metals. It is likely that the repression of transcription in *B. subtilis* does not function as well as in the natural host of the regulation unit (*S. aureus*), causing a higher basal expression level of the luciferase gene and resulting therefore in lower induction coefficients. Indeed, uninduced *B. subtilis* cells

emitted about ten times more light per cell than *S. aureus* cells (data not shown). In addition to being more sensitive to cadmium, *B. subtilis* has a different range of inducers so it could also be used for the detection of zinc. Furthermore, it is an environmental bacteria, with a GRAS (generally regarded as safe) status (NIH guidelines, 1994). This fact is the most important factor in gaining widespread usage and acceptance as a sensor bacteria for field applications.

Freeze-drying of strain RN4220(pTOO24) affected both the sensitivity and the induction efficiency of cells (Fig. 4), whereas it did not affect strain BR151(pTOO24) as much, only the sensitivity was decreased slightly. Further optimization of freeze-drying conditions will be needed before these cells can fully replace freshly cultivated cells. The results with other sensor strains with different plasmids described by us (Lampinen et al., 1995; Tauriainen et al., 1997) suggest that freeze-dried cells can be readily used instead of freshly cultivated cells. This would make the reagent-like usage of the sensor cells for the detection of heavy metals possible. Also, the incorporation of cells into biosensors by immobilization (Åkerman et al., 1990) or entrapping is a viable option.

Despite the fact that bioavailability has been shown to be a major factor in determining metal toxicity (Farrell et al., 1993), bioavailability measurements have not been widely adopted in monitoring of environmental samples. Measurements of total metal content of samples with chemical analysis does not give any information about the speciation of a metal. Yet it might be that only one of the ion species is responsible for the biological effect which the metal has on living organisms and this species could be present only at a minor fraction of the total metal content (Hughes and Poole, 1991). By using the recombinant sensor bacteria, such as the one described here and in our previous studies (Tauriainen et al., 1997; Virta et al., 1995), in addition to chemical analysis it is possible to distinguish bioavailable metal from the total metal content of samples. Due to changes in the environment, an inert, unavailable, metal could become bioavailable and result to unexpected effects. For this reason it could be useful to have a possibility to examine the conditions where a change in bioavailability occurs.

Another application for bioavailability measurements could be useful is bioremediation. Although it is known that the metabolic activity of microbes may result in the modification of the distribution of ion species (Hughes and Poole, 1991), there is still limited knowledge concerning use of microbes in cleaning of environments contaminated by heavy metals. Collard et al. (1994) presented a bioreactor for cleaning of heavy metal contaminated effluents, which was based on the precipitation of metals due to microbial action. This kind of bioreactor can be useful for industrial effluents, but if the metal contamination is already in the environment it would be too expensive to use. Yet, methods based on metal precipitation can only be useful for liquids; in solids, for example soil, methods based on metal solvating could be more appropriate. As a tool for the development of metal bioremediation processes, heavy metal sensing bacteria could play an important role.

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