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Whole-cell biosensors for detection of heavy metal ions in environmental samples based on metallothionein promoters from *Tetrahymena thermophila*

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Summary

Heavy metals are among the most serious pollutants, and thus there is a need to develop sensitive and rapid biomonitoring methods for heavy metals in the environment. Critical parameters such as bioavailability, toxicity and genotoxicity cannot be tested using chemical analysis, but only can be assayed using living cells. A whole-cell biosensor uses the whole cell as a single reporter incorporating both bioreceptor and transducer elements. In the present paper, we report results with two gene constructs using the *Tetrahymena thermophila MTT1* and *MTT5* metallothionein promoters linked with the eukaryotic luciferase gene as a reporter. This is the first report of a ciliated protozoan used as a heavy metal whole-cell biosensor. *T. thermophila* transformed strains were created as heavy metal whole-cell biosensors, and turn on bioassays were designed to detect, in about 2 h, the bioavailable heavy metals in polluted soil or aquatic samples. Validation of these whole-cell biosensors was carried out using both artificial and natural samples, including methods for detecting false positives and negatives. Comparison with other published cell biosensors indicates that the *Tetrahymena* metallothionein promoter-based biosensors appear to be the most sensitive eukaryotic metal biosensors and compare favourably with some prokaryotic biosensors as well.

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Introduction

The importance of metals in living systems is well known, including their role as essential cofactors for many proteins necessary in metabolism and growth (Lovley, 2000). On the other hand, certain metals are among the most abundant, toxic and persistent inorganic environmental pollutants (Hill, 2004). Anthropogenic sources, mainly mining and industrial activities, have substantially increased the heavy metal content in the atmosphere and in many terrestrial and aquatic ecosystems (Peñuelas and Filella, 2002). To monitor and minimize exposure of humans and other organisms to metal pollution, there is a pressing need to develop accurate metal detection assays for potentially polluted environmental samples. While metal concentrations can be measured using molecular recognition or chemical analysis, critical parameters such as bioavailability, toxicity and genotoxicity can only be assayed using living cells. Recently, the concept of whole-cell biosensor (WCB) or bioreporter has been introduced by several authors (D'Souza, 2001; Belkin, 2003; Van der Meer and Belkin, 2010), as a very useful alternative to classical biosensors. A WCB uses the whole prokaryotic or eukaryotic cell as a single reporter incorporating both bioreceptor and transducer elements. In general, living systems to be used as WCBs are cells that are experimentally modified to incorporate the transducer capacity. Two types of bioassays using WCBs may be considered; turn off and turn on assays (Belkin, 2003). In turn on assays a quantifiable molecular reporter is fused to a specific gene promoter, known to be activated by the target chemical or environmental pollutant, such as heavy metals.

The ciliates, which are eukaryotic microorganisms, offer a host of favourable qualities as potential WCBs (Gutierrez *et al.*, 2003; 2004; 2008). In particular, they offer two specific advantages. First, ciliates do not have a cell wall in their vegetative phase, which in other organisms can lower the sensitivity to environmental pollutants as well as delay the cellular response (Martin-Gonzalez *et al.*, 1999; Gutierrez *et al.*, 2003). Second, for several metabolic pathways, ciliates more closely resemble human cells than do bacteria or yeasts, as revealed by genome

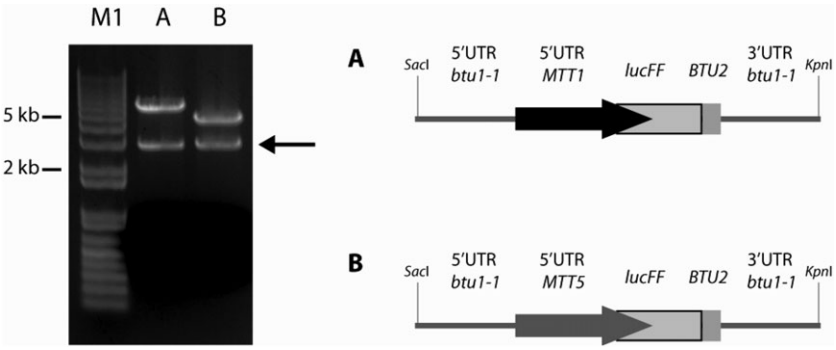

Fig. 1. The pMTT1LucFF (6.3 Kb) and pMTT5LucFF (5.3 Kb) plasmid constructs. Electrophoresis analysis shows the expected *Sac*I/*Kpn*I double digest products (4.5 and 3.5 kb in size, arrows) from plasmids pMTT1LucFF (lane A) and pMTT5LucFF (lane B). M1: size marker M1 Kb (Roche). Features of these constructs are indicated in panels A and B (see the text for explanation). *BTU2*: polyadenylation sequence from *BTU2* gene.

analysis in *Tetrahymena thermophila* and *Paramecium tetraurelia* (Aury *et al*., 2006; Eisen *et al*., 2006). It suggests that ciliates offer, for some ecotoxicological applications, a strong alternative to testing in animals (Gutierrez *et al*., 2003; 2008).

Metallothioneins (MTs) are metal-binding proteins that confer protection from metal toxicity, and expression of the corresponding genes is therefore induced by cell exposure to metals. Five *T. thermophila* genes encode MT isoforms, which are induced primarily by Cd^{2+} (*MTT1, MTT3* and *MTT5*) or Cu^{2+} (*MTT2* and *MTT4*) (Diaz *et al*., 2007; Gutierrez *et al*., 2009). The CdMT genes are expressed at very different levels (*MTT5* >> *MTT1* > *MTT3*) under most stress conditions, including heavy metals (Diaz *et al*., 2007). Consistent with this pattern, the *MTT5* and *MTT1* promoters respond rapidly and strongly to heavy metals (Shang *et al*., 2002; Diaz *et al*., 2007), and the inducibility of the *MTT1* promoter has been exploited to facilitate the regulated overexpression of homologous or heterologous genes in *T. thermophila* (Shang *et al*., 2002; Cole *et al*., 2008).

In this article, we describe two fusions between the *MTT1* or *MTT5* promoters and the eukaryotic luciferase gene (*lucFF*), which, when stably expressed in *T. thermophila*, result in strains that function as heavy metal WCBs, the first such strains to be reported in ciliates. These strains function in turn on bioassays that can rapidly detect bioavailable heavy metals in environmental samples. The *Tetrahymena* WCBs compare favourably with many currently available prokaryotic or eukaryotic cell metal biosensors.

Results

MTT1Luc and MTT5Luc recombinant strains

To create the reporter constructs *MTT1::lucFF* and *MTT5::lucFF*, we placed the eukaryotic luciferase gene (*lucFF*) as a reporter under the transcriptional control of the promoters for the *T. thermophila MTT1* or *MTT5* genes, as described in *Methods*. These constructs were each integrated and driven to fixation at the non-essential *btu1-1* locus in the *T. thermophila* macronucleus to create stable cell lines. The resulting plasmids pMTT1Luc and pMTT5Luc were restriction digested with KpnI and SacI to release the reporter constructs (*MTT1::LucFF* or *MTT5::LucFF*) (Fig. 1), which were then introduced into *T. thermophila* CU522 strain by biolistic bombardment (Cassidy-Hanley *et al*., 1997). The linearized constructs were designed to undergo homologous recombination at the *BTU1* locus, which in the CU522 strain bears a paclitaxel-hypersensitive allele, *btu-1–1* (Gaertig *et al*., 1994). Consequently, the desired recombinants could be selected based on their paclitaxel resistance. These cell lines were named MTT1Luc and MTT5Luc (Table 1). Starting with these paclitaxel-resistant isolated clones, we then obtained strains with complete macronuclear replacement (i.e. homozygous strains for *btu-1–1::*(*MTT1* or *MTT5*)*lucFF*

Table 1. Genotypic and phenotypic features of the recombinant strains MTT1Luc and MTT5Luc.

Strain	Micronuclear genotype	Macronuclear genotype	Macronuclear phenotype
MTT1Luc	*mpr1/mpr1, btu1-1/btu1-1*	*mpr1, btu1-1::MTT1LucFF*	mp-R, pac-R, MTT1Luc, VI
MTT5Luc	*mpr1/mpr1, btu1-1/btu1-1*	*mpr1, btu1-1::MTT5LucFF*	mp-R, pac-R, MTT5Luc, VI

mpr1: 6-methylpurine resistance (mp-R); *btu1-1*: paclitaxel sensitivity; pac-R: paclitaxel resistance; *MTT1LucFF*: luciferase expressed from the *MTT1* promoter; *MTT5LucFF*: luciferase expressed from the *MTT5* promoter; VI: mating type 6.

by taking advantage of phenotypic assortment (Gaertig *et al.*, 1994).

Design of a turn on bioassay using MTT1Luc or MTT5Luc strains as heavy metal WCBs

MTT5Luc cells were grown to log growth phase, transferred to 0.01 mol l^{-1} Tris-HCl pH 6.8 to avoid the metal-chelating activity of organic matter in the culture medium, and incubated with 0.5 μmol l^{-1} Cd^{2+} (we selected this metal concentration because it showed the best luciferase expression levels, without cell mortality). Under these conditions, a 2 h exposure to Cd^{2+} resulted in maximal bioluminescence/luciferase activity values, with no further increase at 3 h (Fig. S1). We therefore used a 2 h incubation time for bioassays.

To compare the sensitivity of measuring luciferase activity in whole cells vs. in cells permeabilized with Triton X-100 and dimethylsulphoxide (DMSO), we compared these *in vivo* and *in vitro* approaches in parallel for both biosensor strains over a range of Cd^{2+} concentrations (Fig. S2). For each individual strain, the *in vivo* and *in vitro* results were quite similar. In addition, both strains showed maximum bioluminescence at similar Cd^{2+} concentrations (0.25 μmol l^{-1}). However, the absolute induction coefficient values of MTT5Luc were more than twice that of MTT1Luc (Fig. S2). These results indicated that MTT5Luc was likely to offer the more sensitive WCB, and that either an *in vivo* or *in vitro* assay could be used to measure luciferase activity. We selected the latter because it allowed us to easily normalize the luciferase activities of different samples with respect to the total cell protein. Furthermore, we found that the *in vitro* flash-type bioluminescence reaction was both stable and prolonged.

Responsiveness of reporter strains: testing a set of heavy metals

To test the responses of the potential WCBs MTT1Luc and MTT5Luc, we exposed these cell cultures to a variety of individual heavy metals (Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, As^{5+} and Hg^{2+}), and combinations of metals. In addition, stress conditions unrelated to heavy metals were also tested, as described further below. Results with individual metals show that *lucFF* induction, when under the control of either the *MTT1* or *MTT5* promoters, is sensitive to both the type of metal and its concentration (Fig. 2). The bioluminescence increases with metal concentration to a maximum level, after which the level decreases because of the metal toxicity (Fig. 2). The majority of metals induce bioluminescence in both strains, including at very low metal concentrations. In general, Cd^{2+} induced the highest bioluminescence output for both recombinant strains (Fig. 2). However, the two strains showed differences in their level of induction. The induced bioluminescence from MTT5Luc (Fig. 2B) ranged between 10- to 60-fold higher than the basal level, while for MTT1Luc the equivalent increase was about 2- to 18-fold (Fig. 2A). Zn^{2+} and Hg^{2+} produced the lowest bioluminescence increases (< 10 times the basal levels) in the MTT5Luc strain (Fig. 2B). On the other hand, MTT1Luc showed small bioluminescent increases in response to Pb^{2+} or As^{5+}, but responded strongly to Cd^{2+}, Zn^{2+}, Cu^{2+} or Hg^{2+} (Fig. 2A). Therefore, the two strains show differential bioluminescence output that is sensitive to both metal type and concentration.

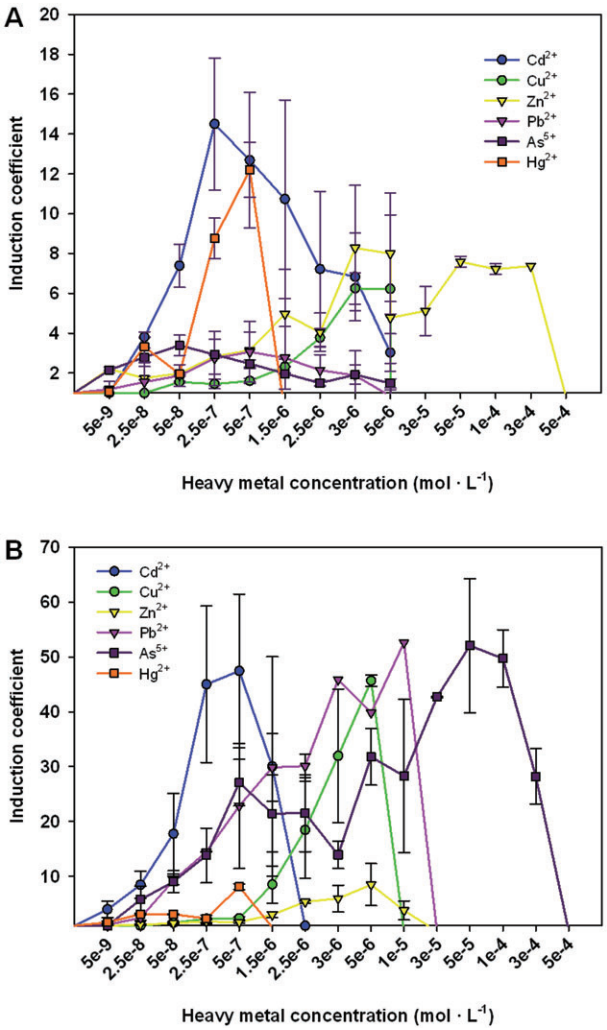

Fig. 2. Response of biosensors to different heavy metals at different molar concentrations, after 2 h exposure in Tris-HCl pH 6.8.
A. MTT1Luc.
B. MTT5Luc.
Data shown represent the average values obtained from three different experiments.

Table 2. Lowest metal concentrations detected by strains MTT1Luc and MTT5Luc.

Metal	MTT1Luc	MTT5Luc	Maximum allowable concentration
Cd^{2+}	$25–50 \times 10^{-9}$	$5–25 \times 10^{-9}$	$8.9 \times 10^{-6}–2.7 \times 10^{-5}$
Cu^{2+}	2.5×10^{-6}	1.5×10^{-6}	$7.9 \times 10^{-4}–2.2 \times 10^{-3}$
Zn^{2+}	0.5×10^{-6}	1.5×10^{-6}	$2.29 \times 10^{-3}–4.6 \times 10^{-3}$
Pb^{2+}	0.5×10^{-6}	50×10^{-9}	$2.4 \times 10^{-4}–1.5 \times 10^{-3}$
As^{5+}	50×10^{-9}	25×10^{-9}	Unspecified
Hg^{2+}	25×10^{-9}	25×10^{-9}	$5 \times 10^{-6}–7.5 \times 10^{-6}$

The minimum detectable metal concentration (mol l^{-1}) for each biosensor, using assay conditions as described in the text. Also listed is the maximum allowable concentration of each heavy metal in soil, as established by the European Directive (86/278/CEE).

The two strains also differed in their sensitivity. The lowest metal concentration that results in detectable bioluminescence by both strains is about 5–50 nmol l^{-1} for non-essential heavy metals (Cd^{2+}, Pb^{2+}, As^{5+} or Hg^{2+}), and about 1 μmol l^{-1} for essential metals (Zn^{2+} or Cu^{2+}) (Table 2). The sensitivity level (lowest metal concentration inducing bioluminescence) of MTT5Luc is higher than that of MTT1Luc, in particular for Cd^{2+}, Pb^{2+} and As^{5+} (Table 2). In all cases, the metals induced reporter gene expression at concentrations lower than their LC_{50}, so neither cell number nor viability was affected.

In general, it is known that metal discharges from mining or industrial practices usually contain more than one heavy metal, so we also analysed the effect of several metal mixtures using MTT5Luc, because this strain showed higher sensitivity and expression levels. In many cases, we observed that bi-metallic mixtures resulted in synergistic induction when compared with the same metals tested individually (Fig. S3).

Specificity of reporter strains

To verify that the *Tetrahymena* WCBs are preferentially sensitive to bioavailable metals, we exposed MTT5Luc to Cd^{2+} that was chelated with Ethylenediaminetetraacetic acid (EDTA) (1 or 10 μmol l^{-1}) (Fig. S4). Chelation resulted in a 60–90% reduction, compared with the non-chelated samples, in the induction of bioluminescence. Useful WCBs for metal contamination should ideally be relatively insensitive to other environmental stresses. We therefore exposed the reporter strains to acidic and basic pH, low and high temperature, and oxidative stress. Likewise, to test if the medium composition affects the biosensor responses under these stress conditions, the treatments (2 h) were carried out in growth medium PP210 or buffer Tris-HCl. None of these stress conditions, or changes in media, resulted in significant induction of bioluminescence (Fig. S5). Taken together, these results indicate that the *Tetrahymena* WCB strains are relatively robust reporters of heavy metal concentration.

*Expression analysis of reporter gene (*lucFF*) by quantitative reverse transcription polymerase chain reaction (RT-PCR)*

To corroborate that bioluminescence changes from both recombinant strains correspond to transcriptional changes of the *lucFF* reporter gene, we used quantitative RT-PCR to analyse this gene under the same conditions of metal stress described above for turn on bioassays (2 h, at one metal concentration). This analysis also permitted us to compare the activities of the two CdMT promoters (*MTT1* and *MTT5*). The results (Fig. S6) were similar to those obtained in bioluminescence turn on bioassays (Fig. 2). The *MTT1* promoter was induced most strongly by Cd^{2+} while the *MTT5* promoter was induced most strongly by Cd^{2+} and Pb^{2+} (Fig. S6).

Validation of MTT1Luc and MTT5Luc as heavy metal WCBs for complex environmental samples

We then tested the ability of MTT5Luc to detect Cd^{2+} that was added to environmental aquatic or soil samples. MTT5Luc showed basal bioluminescence (Fig. 3) when incubated with water samples from a man-made pond, but

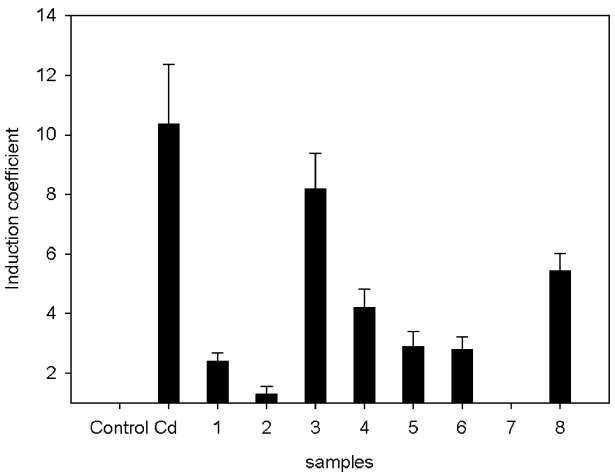

Fig. 3. Response of MTT5Luc to diverse soil samples. The positive control was a culture treated with Cd^{2+} (50 nmol l^{-1}) in Tris-HCl buffer (pH 6.8). Soil samples; 1: Ontario Lake region (Canada) (pH 6.5), 2: Madrid Retiro Park (Spain) (pH 7.5), 3: City park from Stockholm (Sweden) (pH 6.5), 4: City park from Amsterdam (Netherlands) (pH 7.5), 5 and 6: ~11 year old metal polluted samples from Aznalcóllar (Seville, Spain) (pH 7.5) (metal pollution as a result of a pyrite mine spill originated in 1998), 7: Waipoua Forest national park (New Zealand) (pH 6) and 8: sample isolated near sulfur fumaroles at Wai-O-Tapu natural park (New Zealand) (pH 6). All soil extract solutions were diluted 10-fold before use, except sample # 8, which was diluted 1000-fold as a result of its toxicity.

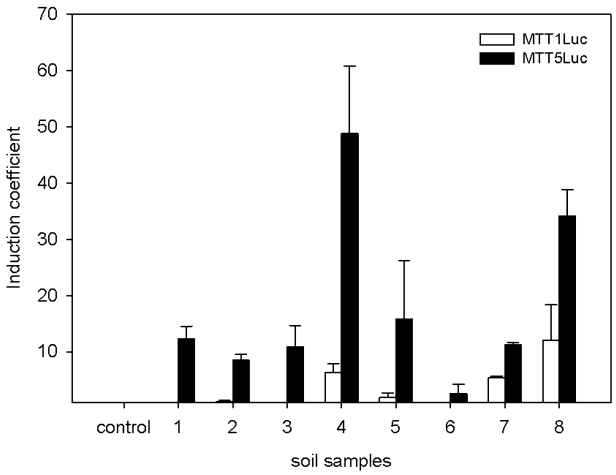

Fig. 4. Comparative response of both biosensors to metal polluted soil samples. MTT1Luc (white bars) and MTT5Luc (black bars). Soil samples (with heavy metal composition determined by spectrometry; see Table S1) were prepared as described in *Methods*. Bioluminescence values are the average of three different experiments.

that bioluminescence increased as expected when we spiked the sample with Cd^{2+}, and this increase was proportional to the amount of Cd^{2+} added. As expected, the bioluminescence increase could be largely eliminated if EDTA was also present (Fig. S7).

Likewise, we tested MTT5Luc against a variety of environmental samples, some of which were known to contain metal pollutants (Fig. 3). Among the varied samples, three (#1: Ontario Lake, Canada; #2: Madrid Retiro Park, Spain, and; #7: Waipoua Forest National Park, New Zealand) did not induce significant bioluminescence in MTT5Luc, suggesting that those soils contained little or no bioavailable metals (Fig. 3). In contrast, MTT5Luc showed substantial induction of bioluminescence after exposure to two soil samples (#3: Stockholm, Sweden city park; #8, Wai-O-Tapu, New Zealand) with lower but significant induction seen in additional samples (#4, Amsterdam, Netherlands, and; #5 and 6, Aznalcóllar, Seville, Spain).

Both WCBs (MTT1Luc and MTT5Luc) were likewise exposed to environmental samples whose metal pollution had been determined by conventional analytic methodology, i.e, the methods specified by recent legislation. Consistent with its high sensitivity, the MTT5Luc strain reported the presence of metals in all samples, with the strongest bioluminescence induced by the most metal-polluted sample (#4) (Fig. 4). The MTT1Luc strain showed induced bioluminescence in response to a subset of the samples (# 4, 7 and 8) (Fig. 4).

Analysing toxic samples by exploiting basal expression

A potential weakness of the WCB approach is that highly polluted samples may be too toxic to support cell viability during the assay period, and therefore produce false negative results. We can recognize such samples because they will suppress not only induced but also basal expression of the reporter genes. Such toxicity could then be confirmed by sample dilution, which should restore the basal and induced responses (see sample #8 in Fig. 3).

Discussion

The metal inducibility of MT genes can serve as a good reporter for the presence of metals in environmental samples (Newman and Unger, 2003). Accordingly, MTs have been included as molecular biomarkers by the European Union and used in environmental assessment programmes (Mathiessen, 2000). While one approach is to detect MT transcripts or protein levels, an advantageous alternative approach is to create transgenic organisms that incorporate a metal-induced promoter (e.g. MT promoter) that is fused to a reporter gene, thereby creating a novel strain that can function as a WCB (Magrisso *et al.*, 2008; Hynninen and Virta, 2010). In *Tetrahymena* as in other organisms, MT gene expression is primarily induced by metal exposure (Dondero *et al.*, 2004; Fu and Miao, 2006; Diaz *et al.*, 2007; Amaro *et al.*, 2008; Guo *et al.*, 2008). The promoters from the *Tetrahymena MTT1* and *MTT2* genes have served as good tools to drive overexpression of homologous or heterologous genes in a metal-inducible fashion (Shang *et al.*, 2002; Boldrin *et al.*, 2008). We have now further exploited these advantageous features and obtained recombinant *T. thermophila* strains incorporating stable macronuclear constructs (*MTT1::lucFF* in MTT1Luc and *MTT5::lucFF* in MTT5Luc) to be used as metal WCBs.

In addition to the specific gene fusion that is incorporated in a WCB, the performance of such a strain will depend on factors such as the assay medium and time of exposure to the inducer, as well as to cell culture parameters such as culture density and growth phase (Liao and Ou, 2005). We designed a turn on bioassay guided by the practical considerations of obtaining a maximum response in the shortest time. We relied on cultures in exponential growth phase (with a cell number $\sim 1–3 \times 10^5$ cells ml^{-1}) to match the conditions of prior studies of *MTT1* and *MTT5* gene expression (Diaz *et al.*, 2007), and because *Tetrahymena* show maximal sensitivity to Cd^{2+} in growth phase (Larsen, 1989). The turn on assays were performed on cells in Tris-HCl, rather than in the PP210 medium in which they were grown, to ensure that metals remained in a bioavailable state. Because MTT5Luc showed maximal response to Cd^{2+} in 2–3 h (Fig. S1), we selected 2 h as a practical choice for the incubation time of all turn on bioassays.

The substrate for eukaryotic luciferase, D-luciferin, is membrane-permeant only in its protonated form (pH 5),

and at neutral pH crosses the plasma membrane very slowly. For this reason most luciferase-based bioassays are performed using cell extracts (Van der Meer *et al*., 2004) or with permeabilized cells (Lagido *et al*., 2001). Interestingly, we found that luciferase activity in MTT1Luc and MTT5Luc could be measured as efficiently in intact viable cells as in permeabilized cells, and we observed similar induction with these *in vivo* and *in vitro* approaches. This indicates that uptake of luciferin in *Tetrahymena* is more efficient than in other eukaryotic model systems that have been used as cellular biosensors, because luciferase assays in WCBs based on *Saccharomyces cerevisiae* or *Caenorhabditis elegans* required that cells be first permeabilized (Hollis *et al*., 2000; Lagido *et al*., 2001). One possible explanation is that, luciferin in the medium may be taken up by fluid-phase endocytosis or phagocytosis, which are very active processes in *Tetrahymena*, and would then be transported into acidifying vesicles, whose pH would facilitate rapid transport across the vesicle membrane into the cell cytoplasm. Whatever the mechanism involved, the efficient uptake of D-luciferin by live *Tetrahymena* makes this organism a more flexible WCB than other established eukaryotic biosensors.

In both *Tetrahymena* strains, all of the metals tested induced luciferase expression at concentrations lower than their respective LC_{50} levels (Gallego *et al*., 2007). While both MTT1Luc and MTT5Luc are therefore effective WCBs, we noted important differences in their responses. Overall, bioluminescence output is higher in MTT5Luc than in MTT1Luc. MTT1Luc showed the strongest response to Cd^{2+}, Hg^{2+}, Zn^{2+} and Cu^{2+}, with less sensitivity to Pb^{2+} and As^{5+}, while MTT5Luc responds strongly to Pb^{2+}, As^{5+}, Cd^{2+} and Cu^{2+}, and more weakly to Zn^{2+} and Hg^{2+} (Fig. 2). In general, these results are consistent with qRT-PCR analysis of the two MT genes after similar metal treatment (Diaz *et al*., 2007), and are also consistent with the previous observation that the LC_{50} values for non-essential metals are lower than those of essential metals (Gallego *et al*., 2007).

Despite these notable differences, reporter constructs bearing fusions to each of the two MT promoters failed to discriminate between different types of metals. Like the majority of WCBs (Magrisso *et al*., 2008; Ivask *et al*., 2009), *Tetrahymena* strains respond to a variety of heavy metals. Because contaminated ecosystems are often polluted with a mixture of metals rather than a single one (Preston *et al*., 2000; Fairbrother *et al*., 2007) and thus various interactions (additive, synergistic or antagonistic) can take place. For that reason, one valuable aim of environmental biomonitoring may be to determine the overall toxicity of a sample, rather than the specific metals present. For the latter, chemical or physical methods are still necessary to determine exactly how much of each metal species is present in a sample.

Our results under a wide range of conditions indicate that the *Tetrahymena* WCB strains give highly reproducible results, and therefore clonal variation (Brehm-Stecher and Johnson, 2004) that might arise over long periods of continuous culture did not present a practical problem. Moreover, the low but detectible basal luciferase expression in these strains can be used to detect false negative results produced by highly toxic samples. Another important point is the ability to detect potential false positive results. Although both biosensors only showed significant bioluminescence increases in response to heavy metals and not to other analysed stressors, there may be other, as yet untested, environmental conditions that induce bioluminescence in these WCBs. We found that induction as a result of heavy metals could be dramatically reduced by pre-chelating the samples with EDTA, and this simple test could be used to distinguish between metal- and potential non-metal-dependent induction by environmental samples.

About 85% of WCBs for metals are based on genetically modified bacteria (Magrisso *et al*., 2008), while ~15% are based on eukaryotes (Walmsley and Keenan, 2000). Two eukaryotic microorganisms have been used, the yeasts *S. cerevisiae* and *Hansenula polymorpha* (Shetty *et al*., 2004; Park *et al*., 2007). The majority of WCBs respond to two or more metals, although several show greater specificity (Corbisier *et al*., 1999; Tom-Petersen *et al*., 2001; Ivask *et al*., 2009). In Table S2, we compare the sensitivity levels of *Tetrahymena* MTT1Luc and MTT5Luc with those of established eukaryotic or prokaryotic WCBs (see Table S2 for references). Both *Tetrahymena* strains are at the top of the ranking for As^{5+} sensitivity ($25–50 \times 10^{-3}$ μmol l^{-1}). For Zn^{2+}, MTT1Luc shares first place with the bacterium *Synechococcus* (0.5 μmol l^{-1}) while MTT5Luc (1.5 μmol l^{-1}) is in third, and both are more sensitive than the *C. elegans* Zn biosensor. Likewise, MTT5Luc and MTT1Luc are in the third and fifth place for Cd^{2+} sensitivity, and MTT5Luc is ~200-fold more sensitive than the *S. cerevisiae* WCB for Cd^{2+}. MTT1Luc and MTT5Luc are the only eukaryotic members of the top 10 sensors for either Hg^{2+} or Pb^{2+}. Finally, MTT5Luc and MTT1Luc occupy fourth and fifth place in Cu^{2+} sensitivity; for this metal, a WCB based on a *S. cerevisiae* CuMT gene promoter (Shetty *et al*., 2004) is about 3–5 times more sensitive than the *Tetrahymena* strains. In the future, *Tetrahymena* WCBs that are more sensitive to Cu^{2+} could be engineered by exploiting one of the two known identical CuMT genes in this microorganism, *MTT2* and *MTT4* (Diaz *et al*., 2007; Boldrin *et al*., 2008). Overall, the comparison suggests that the *Tetrahymena* WCBs are often the most sensitive microbial eukaryotic metal biosensors, and sometimes exceed available bacterial WCBs as well.

A potential weakness of the WCB approach is that highly polluted samples may be too toxic to support cell

viability during the assay period, and therefore produce false negative results. We can recognize such samples because they will suppress not only induced but also basal expression of the reporter genes. Such toxicity could then be confirmed by sample dilution, which should restore the basal and induced responses (see sample #8 in Fig. 3).

Although a large number of metal WCBs have been developed, most have not been evaluated using natural samples (Rodriguez-Mozaz *et al*., 2006; Van der Meer and Belkin, 2010). We demonstrate that MTT1Luc and MTT5Luc can detect bioavailable heavy metals in natural soil or aquatic samples, including samples with metal concentrations lower than the maximum values established by the European Directive (86/278/CEE) (Table 2). Moreover, neither cell viability nor luciferase expression was significantly affected by acid (5) or basic pH (9). The performance of bacterial heavy metal biosensors has been reported at pH between 5.5 and 8, depending on the host bacteria (Tauriainen *et al*., 1999). The broad pH tolerance of the *Tetrahymena* WCBs is advantageous because pH is the major parameter determining metal bioavailability in environmental samples (Fairbrother *et al*., 2007).

Although some WCBs have been commercialized (Corbisier *et al*., 1999) and are used in European laboratories, there is as yet no legislative recognition of their potential contribution for assessing bioavailable heavy metal contamination (Magrisso *et al*., 2008), and they are not included in the recent United States Environmental Protection Agency framework for metal risk assessment (Fairbrother *et al*., 2007). However, this situation may change as well-characterized WCBs become increasingly available and their advantages better known, not as substitutes for conventional spectroscopic analysis but rather to be used in combination (Harkins *et al*., 2004). For example, WCBs might be used as a 'first filter' to detect metals in natural samples, and help to establish a prioritized listing of metal polluted areas for posterior treatment or bioremediation, as well as used to monitor the bioremediation or cleanup. Because both the instrumentation and assay costs for WCBs like those in *Tetrahymena* are much lower than for conventional spectroscopic analysis, the expanded use of WCBs might facilitate larger surveys than would otherwise be practical.

Finally, from this work we conclude the following points: (i) Luciferase activity in MTT1Luc and MTT5Luc can be measured as efficiently in intact viable cells as in permeabilized cells, indicating that uptake of D-luciferin in *Tetrahymena* is more efficient than in other eukaryotic cell biosensors like *S. cerevisiae* or *C. elegans*, where luciferase assays require cell permeabilization. This difference may make *Tetrahymena* a more flexible system for some applications; (ii) Both WCB strains (MTT1Luc and MTT5Luc) show differential responses to a variety of environmentally important metals, because of the properties of each MT promoter. The differences in response are consistent with previous analysis of the transcription of these MT genes; (iii) The low but detectible basal luciferase expression in these strains can be used to detect false negative results produced by highly toxic samples, and methods are also available to detect potential false positives; (iv) The comparison with other microbial metal cell biosensors suggests that for many applications *Tetrahymena* WCBs offer the most sensitive metal biosensors that are available among eukaryotic microbes, sometimes exceeding the available bacterial WCBs as well; (v) Like other WCBs, *Tetrahymena* WCBs do not distinguish among different heavy metals (because *Tetrahymena* CdMT promoters, although preferentially respond to Cd, they are also inducible by other metals). They detect bioavailable heavy metals, with a high and differential sensitivity, in both artificial and natural samples.

Experimental procedures

Cell culture and stress treatments

The *T. thermophila* strain CU522 (Gaertig *et al*., 1994) (*mpr1/mpr1, btu1-1/btu1-1*; *mpr1, btu1-1,* mp-r, ory-r, pac-s, VI) was kindly supplied by J. Gaertig (University of Georgia, USA). The strain is homozygous for *btu1-1*, a mutant β-tubulin allele that is positively and negatively selectable and therefore allows easy selection of transformants with transgenes that are targeted for recombination at that locus. It was grown axenically in SPPA medium (2% proteose peptone (Difco), 0.1% yeast extract (Difco), 0.2% glucose (Sigma), 0.003% Fe-EDTA (Sigma), supplemented with 250 μg ml^{-1} penicillin G and streptomycin sulfate (Sigma) and 0.25 μg ml^{-1} amphotericin B (Sigma) or PP210 medium (Diaz *et al*., 2007), and maintained at 30 ± 1°C. Culture media and all reagents were made in Milli-Q (Millipore) grade water. For heavy metal experiments and sample preparation see Appendix S1.

Construction of transformation plasmids

The pBt(Δ7)Luc plasmid (kindly provided by D. Romero, University of Minnesota, USA) includes a luciferase reporter (*lucFF* gene) transcribed from the *BTU1* promoter and with a *BTU2 3′* flank (Gaertig *et al*., 1994). This plasmid was used to generate the *MTT1::lucFF* or *MTT5::lucFF* constructs. See Appendix S1 for more information on construction of transformation plasmids.

Biolistic transformation

Tetrahymena thermophila strain CU522 was grown in 50 ml culture (SPPA medium) to ~2–4 × 10^5 cell ml^{-1}, and starved for 12–18 h in DMC buffer (1/10 dilution Dryl buffer, supple-

mented with 0.1 mmol l^{-1} $MgCl_2$ and 0.5 mmol l^{-1} $CaCl_2$). The cells were pelleted by centrifugation and resuspended in 1 ml DMC buffer. See Appendix S1 for details on biolistic transformation.

DNA and RNA isolation, standard PCR and quantitative real time RT-PCR

Tetrahymena DNA was isolated from 50 ml exponential cultures as described in Hamilton and Orias (2000). Bacterial plasmids were isolated with the QIAprep Spin Miniprep kit (Qiagen). Total RNA was isolated according to the protocol supplied by the RNeasy Mini kit (Qiagen). To remove possible DNA contamination, all samples were treated with DNase I (RNase free) (Ambion). Total RNA samples were analysed using denaturing 1.2% agarose gels, according to Sambrook and Russell (2001). See Appendix S1 for specifications on PCR and quantitative RT-PCR.

Luciferase activity assays

Tetrahymena thermophila transformant strains (MTT1Luc and MTT5Luc) were grown in PP210 medium to densities of $1–3 \times 10^5$ cells ml^{-1} and pelleted (3 min at 1100 *g*), then cells were washed and resuspended in 0.01 mol l^{-1} Tris-HCl buffer pH 6.8. 5 ml aliquots of cells were transferred to sterile tubes and exposed, during 2 h at 30°C, to the different heavy metal concentrations, other stressors or natural soil or aquatic samples. Luciferase activity was quantified in three different ways: *in vitro* from cell extracts, *in vivo* from permeabilized cells to D-luciferin and *in vivo* from cells without any previous treatment. For *in vitro* quantification, we cold-lysed cells with 350 µl of 1x CCLR (Cell Culture Lysis Reagent, Promega). The lysates were centrifuged at 16 250 *g* for 2 min at 4°C, and supernatants used immediately to quantify luciferase activity. The extracts were adjusted to a protein concentration of 0.5 mg ml^{-1}, as determined using the Bradford method according to Sambrook and Russell (2001). Luciferase activity was determined with the Luciferase Assay Kit (Promega), using 20 µl of Tris-HCl buffer cell suspension with 100 µl of luciferase assay reagent (Promega) containing D-luciferin, and making 10 s measurements with a Berthold LB 9509 Junior (Berthold) luminometer. To measure activity in permeabilized cells, we pelleted cells in Tris-HCl at 500 *g* (2 min) and immediately suspended the pellet in 5 ml of 0.2% DMSO + 0.01% Triton X-100 (at room temperature). The cell suspension was centrifuged (1 min at 500 *g*) and all but about 500 µl of the supernatant removed. From this suspension, 20 µl of aliquots was taken and mixed with 100 µl of luciferase assay reagent (Promega) containing D-luciferin, and bioluminescence was quantified after ~1 min. For *in vivo* quantification without cell permeabilization, 20 µl of Tris-HCl buffer cell suspension was added to 100 µl of luciferase assay reagent (Promega) with D-luciferin, and after ~1 min the bioluminescence was quantified. Bioluminescence level was expressed as the ratio; light emission by the cells exposed to metal/light emission by the cells without metal exposure (basal expression), and it is named 'Induction coefficient'.

Patents

The bioreporter constructs pMTT1LucFF and pMTT5LucFF have been accepted by the Spanish Office of Patents (Ministerio de Industria, Turismo y Comercio) for patenting, with the reference numbers P200901622 and P200901621 respectively.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Time course of MTT5Luc response to Cd^{2+} (5×10^{-7} mol L^{-1}). Data represent the average values from two different experiments.

Fig. S2. Comparative bioluminescence response, at different Cd^{2+} (mol L^{-1}) concentrations. Whole-cells (*in vivo* bioassay) (black line), cell extracts (*in vitro* bioassay) (black bars) or permeabilized cells (grey bars) of both MTT1Luc (A) or MTT5Luc (B) biosensors.

Fig. S3. MTT5Luc response to different metal mixtures. (A): $Cd^{2+} + Cu^{2+}$, (B): $Cd^{2+} + Zn^{2+}$, (C): $Cd^{2+} + Pb^{2+}$, (D): $Cu^{2+} + Zn^{2+}$. Cd^{2+} or Cu^{2+} molar concentrations: 0 mol L^{-1} (white bars), 2.5×10^{-7} mol L^{-1} (grey bars), 5×10^{-7} mol L^{-1} (black bars). Bioluminescence measures were carried out *in vitro*. Heavy metal interactions are pointed out on the corresponding bars as A (additive) or S (synergistic).

Fig. S4. The MTT5Luc response depends on Cd^{2+} bioavailability. Control (black circles): Cells were exposed to Cd^{2+} at the molar concentrations shown, for 2h in Tris-HCl buffer. EDTA (1) and (2): 4h prior to mixing with cells, the Cd^{2+} was chelated by addition of 1 μmol L^{-1} EDTA (white circles) or 10 μmol L^{-1} EDTA (black triangles).

Fig. S5. Biosensor response to different non-metal stressors. (A) MTT1Luc in PP210 medium. (B) MTT1Luc in Tris-HCl buffer. (C) MTT5Luc in PP210. (D) MTT5Luc in Tris-HCl buffer. All treatments were during 2h. PQ: Paraquat.

Fig. S6. Expression analysis of reporter gene (*lucFF*) by quantitative RT-PCR. Relative expression of the reporter gene *lucFF* from MTT1Luc (white bars) and MTT5Luc (black bars), obtained by qRT-PCR of cells exposed (2 h) to diverse heavy metals (5×10^{-7} mol L^{-1}) in Tris-HCl buffer (pH 6.8). Gene expression levels are shown relative to an untreated control (which is set at 1 ± 0.0), and *ATU1* (α-tubulin) gene expression was used to normalize all samples. Each bar represents the average of two independent experiments. (*): Significantly different from the control at $p < 0.05$.

Fig. S7. MTT5Luc response to Cd^{2+} added to a non-contaminated (by heavy metals) natural aquatic sample. Natural aquatic sample + Cd^{2+} (black bars). Control (0 Cd^{2+}): sample without added Cd^{2+}. Cd^{2+} + 10 μmol L^{-1} EDTA (grey bars).

Table S1. Total metal content of soil samples used in experiments shown in Fig. 4.

Table S2. Ranking sensitivity to different heavy metals by previously reported eukaryotic or prokaryotic WCBs and the MTT1Luc and MTT5Luc strains.

Appendix S1. Experimental procedures.

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