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METHODS AND PROTOCOLS

Simultaneous detection of bioavailable arsenic and cadmium in contaminated soils using dual-sensing bioreporters

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Abstract Whole-cell bioreporters (WCBs) have attracted increasing attention during the last few decades because they allow fast determination of bioavailable heavy metals in contaminated sites. Various WCBs to monitor specific heavy metals such as arsenic and cadmium in diverse environmental systems are available. However, currently, no study on simultaneous analysis of arsenic and cadmium has been reported, even though soils are contaminated by diverse heavy metals and metalloids. We demonstrated herein the development of dual-sensing WCBs to simultaneously quantify bioavailable arsenic and cadmium in contaminated sites by employing the promoter regions of the *ars* and *znt* operons as separate metal-sensing domains, and *egfp* and *mcherry* as reporter genes. The dual-sensing WCBs were generated by inserting two sets of genes into *E. coli* DH5α. The capability of WCBs was successfully proved to simultaneously quantify bioavailable arsenic and cadmium in amended Landwirtschaftliche Untersuchungs und Forschungsanstalt (LUFA) soils, and then, it was applied to contaminated field soils collected from a smelter area in Korea. As a result, it was noticed that the bioavailable portion of cadmium was higher than that of arsenic while the absolute amount of bioavailable arsenic and cadmium level was opposite. Since both cadmium and arsenic were assessed from the same *E. coli* cells, the data obtained by using dual-sensing WCBs would be more efficient and convenient than that from comparative WCB assay. In spite of advantageous aspects, to our knowledge, this is the first report on a dual-sensing WCB for rapid and concurrent quantification of bioavailable arsenic and cadmium in contaminated soils.

Keywords Whole-cell bioreporter · Dual-sensing · Soil · Heavy metal · Bioavailability

Introduction

Heavy metals are naturally present in soils, but in case of excessive inflow and accumulation in the environment, they are one of the major pollutants and pose serious ecological and health risks. Generally, heavy metal contents are monitored and analyzed by using analytical instruments. However, current methods provide the total amount of heavy metals in an environment, rather than the bioavailable amount, which can provide more relevant information about their biological effects. Moreover, traditional instrumental analysis is time-consuming and expensive. To overcome these disadvantages, bacterial cell-based bioreporters have drawn attention in the past few decades as an alternative for monitoring the bioavailability of heavy metals and metalloids.

Whole-cell bioreporters (WCBs) are genetically engineered bacterial cells that produce a signal in response to target substrates. In general, WCBs contain a sensing element that recognizes a specific pollutant and a reporter gene that indicates the effects of the pollutant on the bacterial strains (Corbisier et al. 1999; Robbens et al. 2010;

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Sorensen et al. 2006; Su et al. 2011; van der Meer and Belkin 2010). Generally, metal-sensing WCBs have been constructed by fusing the gene promoters of metal-inducible operons such as *merR*, *zntA*, *chrB*, *copA*, and *arsR* from diverse microorganisms as sensing elements for mercury, zinc, chromium, copper, and arsenic, respectively, to genes encoding proteins such as LacZ, luciferase, and green fluorescent protein (GFP) as signaling elements (Baumann and van der Meer 2007; Branco et al. 2013; Brocklehurst et al. 1999; Diesel et al. 2009; Gireesh-Babu and Chaudhari 2012; Ivask et al. 2009; Magrisso et al. 2008; Priyadarshi et al. 2012; Riether et al. 2001; Selifonova et al. 1993). Because a wide array of sensing and signaling elements is available, a large number of WCBs can be generated by combining these. Similar to other heavy metal-specific WCB systems, dual-sensing WCBs can be established by inserting two sets of sensing-signaling elements into a single bacterial strain. Previously, WCBs using dual colors have been reported, in which one set was used to detect a toxic substrate, and the other was used as an internal standard for bacterial cell viability (Mirasoli et al. 2002; Roda et al. 2011). Moreover, dual-color *E. coli* bioreporters for genotoxicity and toxic cellular stress based on *recA*::*egfp* and *grpE*::*dsRedExpress* constructs, respectively, have been reported (Belkin 2003). However, these reporters monitor broad effects of diverse pollutants rather than quantify specific toxic materials in a contaminated environment. Hence, it would be more efficient and useful to have WCBs that specifically, quantitatively, and simultaneously determine multiple toxic materials in contaminated sites.

Among the major heavy metals responsible for soil contamination, arsenic and cadmium are known to inhibit plant growth and photosynthesis and to accumulate in various crop plants (Abedin et al. 2002; Liang et al. 2005; Morrison 1969; Williams and David 1976). Moreover, they are potentially toxic materials with adverse effects on human health (Abernathy et al. 2003; Garcia-Morales et al. 1994; Tchounwou et al. 2004). Therefore, WCBs for monitoring bioavailable arsenic and cadmium have been intensively developed; however, these WCBs showed a specific response to one metal or a broad response to multiple metals (Jusoh and Wong 2014; Kaur et al. 2015; Riether et al. 2001; Tao et al. 2013). In this study, we developed and applied arsenic- and cadmium-specific dual-sensing WCBs to simultaneously determine the bioavailability of both metals in polluted soils in a smelter area in Korea. The WCBs effectively showed differential responses to arsenic and cadmium in soils contaminated with various metals. To the best of our knowledge, this is the first dual-sensing WCB to monitor and quantify the bioavailability of two heavy metals in a contaminated site simultaneously. We believe that monitoring multiple pollutants simultaneously in contaminated environmental systems is pertinent to the future direction of WCBs.

Materials and methods

Bacterial strain, chemicals, and contaminated soils

E. coli DH5α was used for plasmid cloning and used as the host bacterial strain of whole-cell bioreporters (WCBs). The chemicals AsCl3, Na2HAsO4, CdCl2, K2Cr2O7, CuCl2·2H2O, HgCl2, NiCl2, PbCl2, and ZnCl2 used for heavy-metal specificity tests were purchased from Sigma Aldrich. Landwirtschaftliche Untersuchungs und Forschungsanstalt (LUFA) 2.2 standard soil (LUFA Speyer, Germany) was used to prepare arsenic- and cadmium-amended soil samples. Soil samples from fields contaminated with both arsenic and cadmium were collected at 6 different locations around an old smelter site.

Construction of the dual-sensing WCBs

The promoter region of the *ars* operon (*ars-pr*), a 110-bp DNA fragment, was amplified by PCR from genomic DNA of *E. coli* DH5α, as a sensing element for arsenic. A 170-bp fragment upstream of the *znt* operon, containing the promoter region (*znt-pr*), was employed as a sensing element for cadmium. Both sensing elements were inserted into *Bgl*II and *Xba*I restriction sites of the pET21(a) vector to replace the T7 promoter. The *egfp* and *mcherry* reporter genes were cloned downstream of the sensing elements into the *Bam*HI and *Xho*I restriction sites to generate pET_ars-pr-eGFP and pET_ars-pr-mCherry, and pET_znt-pr- eGFP and pET_znt-pr-mCherry. The *znt-pr::egfp* and *znt-pr::mCherry* fragments were cut out by *Bgl*II/*Xho*I digestion and inserted to pCDFDuet-1 plasmid that contains a spectromycin resistance gene to generate pCDFDuet_znt-pr-eGFP and pCDFDuet_znt-pr-1 mCherry, respectively. Two sets of plasmids were transformed into *E. coli* DH5α and the dual-sensing WCBs were selected on agar plates containing 100 μg/mL of both ampicillin (pET21(a)) and spectromycin (pCDFDuet).

Whole-cell bioreporter assay

To analyze the bioavailable portion of As(III) and Cd(II), the dual-sensing WCBs were grown at 37 °C in the shaking incubator overnight, and then 100 μL of culture was transferred to 5 mL of Lysogeny Broth (LB) media. When the cell density was reached to 0.4 of OD_{600}, the contaminated samples were added. After 1 and 2 h of exposure duration, 1 mL of culture was collected for the analysis. In case of aqueous samples, 1 mL of WCB culture was resuspended by 50 mM Tris-HCl (pH 7.4) containing 160 mM of KCl, and then measured the intensity of eGFP and mCherry by FS-2 fluorescence spectrometer (Scinco, Korea). In case of contaminated soil, two steps of centrifugation were employed to remove soil particles. Briefly, 1 mL of WCBs exposed to contaminated soils was

centrifugated for 1 min at 500 rpm, and then, supernatant was transferred to new tubes for second centrifugation for 1 min at 10,000 rpm. The cell pellets were resuspended by same buffer used for soil solution and applied to fluorescence spectrometer. The bandwidth of excitation and emission was set at 5 nm, and 470/510 and 580/610 nm were used as excitation/emission wavelengths for eGFP and mCherry, respectively. The response of WCBs toward bioavailable metal and metalloid ions was represented as an induction coefficient defined as [emission intensity of WCB exposed to heavy metal ions]/[emission intensity of WCB without heavy-metal exposure].

Heavy-metal selectivity of the dual-sensing WCBs

The heavy-metal selectivity of the dual-sensing WCBs harboring *ars-pr*::*mCherry*/*znt-pr-eGFP* and *ars-pr*::*eGFP*/*znt-pr*::*mCherry* toward heavy metals and metalloids known as major soil contaminants, including As(III), As(V) Cd(II), Cr(VI), Cu(II), Hg(II), Ni(II), Pb(II), and Zn(II), was investigated. Five milliliters of the WCBs were treated with heavy metal and metalloid ions at a final concentration of 1.0 and 10.0 ppm (μg/mL) prepared from stock solutions. The emission intensity of WCBs exposed to metal and metalloids ions was measured by fluorescence spectrometer after 1 and 2 h of exposure. The response toward heavy metal(loid)s was represented as an induction coefficient.

Differential responses of dual-sensing WCBs toward As(III) and Cd(II)

The dual-sensing WCBs were grown in LB media containing 100 μg/mL of ampicillin and spectromycin medium and exposed to As(III) and Cd(II) alone and in combination. Because the response rate of the promoter and the maturation time of the reporter genes were different, the WCB harboring *ars-pr*::*mCherry*/*znt-pr-eGFP* was treated with 0–10 μg/mL of As(III) and 0–5 μg/mL of Cd(II), and the WCB harboring *ars-pr*::*eGFP*/*znt-pr*::*mCherry* was treated with 0–0.5 μg/mL of As(III) and 0–30 μg/mL of Cd(II). The expression of reporter genes of WCBs induced by arsenic and cadmium exposure was represented as an induction coefficient using the WCBs without arsenic and cadmium treatment as references. In addition, the single metal sensing WCBs were generated by transforming each of pET_ars-pr-mCherry and pCDFDuet_znt-pr-eGFP to compare the capability to detect arsenic and cadmium to dual-sensing WCBs.

Fluorescence microscopic analysis of dual-sensing WCBs exposed to As(III) and Cd(II)

Fluorescence images of the dual-sensing WCB were taken by using a fluorescence microscopy (Olympus BX51; Olympus, Tokyo, Japan). Expression of mCherry induced by As(III) was detected using a filter set with excitation at 510–550 nm and emission at 590 nm (Olympus U-MWG2 filter unit). Cd(II)-induced eGFP expression was detected using a filter set with excitation at 460–495 nm and emission at 510 nm (Olympus U-MWIB3 filter unit).

Analysis of arsenic and cadmium bioavailability in contaminated soils

The amount of bioavailable As(III) and Cd(II) was measured in LUFA soils amended with both metals and contaminated site soils from a smelter area. LUFA soil samples were spiked with As(III)/Cd(II) at 200/10, 100/50, and 100/100 μg/g, and stored in a dark place for 7 days for aging before quantification using the dual-sensing WCBs. Field soil samples collected from six locations around a smelter site were prepared by drying in the air, grinding, and sieving before the WCB analysis. First, the soil samples were dried in a greenhouse for 2 weeks. Then, the aggregated soil was ground with a hammer and passed through a 2-mm size sieve. Soil samples (0.25 g) were added to 5 mL of WCB cells, and the induction coefficient after 1 h exposure was measured, and the amount of bioavailable metals was calculated from the standard curves. The standard curves were obtained by following the same procedure reported previously (Yoon et al. 2015). Briefly, 0–1 μg/mL of As(III) and 0–1 μg/mL of Cd(II) was spiked as a reversed order to As(III) to minimize cross-selectivity effects and heavy-metal toxicity caused by high metal concentration to 5 mL of WCB with 0.25 g of LUFA soils. Then, the induction coefficient of each test unit was measured after 2-h exposure and plotted against the concentration of the spiked metal concentration. The standard curves were fit to a single rectangular hyperbola with two parameters ($f = a \times x/(b + x)$) by nonlinear regression using SigmaPlot. The bioavailable arsenic and cadmium were determined by measuring the induction coefficients of mCherry and eGFP from dual-sensing WCBs exposed to contaminated soils, and the amounts of bioavailable arsenic and cadmium were converted to micrograms per 1 g of soil. The bioavailable portions of arsenic and cadmium were calculated as the ratio to the total amount of the corresponding metal in the samples, which was determined by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, JY 138; Ultrace, Jobin Yvon, France). For the ICP-AES analysis, 1 g of soil was dissolved in mixed acid (nitric acid/hydrochloric acid = 3:1) in Teflon bomb and left to react on a temperature-controlled hot plate (THB-1024 HOT BLOCK Digester; Tekton, Korea) at 120 °C for 48 h. The mixture was diluted with 20 mL of distilled water and filtered through filter paper (8-μm pore size, WHATMAN #2; Whatman International, Maidstone, Kent, UK). Standard solutions (1000 mg/L; Sigma-Aldrich, St. Louis, MO, USA) were used for calibration.

Additionally, the total amounts of arsenic, cadmium, copper, nickel, lead, and zinc in the contaminated site soils were determined by ICP-AES.

Results

Selectivity of the dual-sensing WCBs toward As(III) and Cd(II)

The *E. coli*-based dual-sensing WCBs investigated in present study harbored the plasmids containing the promoter regions of the *ars* operon (*ars-pr*) and the *znt* operon (*znt-pr*) as sensing elements for arsenic and cadmium, respectively, with reporter genes encoding eGFP and mCherry. In fact, WCBs employing *ars-pr* and *znt-pr* have been previously reported as bioreporters detecting bioavailable arsenic and diverse divalent heavy metals such as Zn(II), Pb(II), and Cd(II), respectively (Abernathy et al. 2003; Belkin 2003; Roda et al. 2011; Rowe et al. 2005; Wuana and Okieimen 2011). To verify dual-metal specificity, the WCBs harboring *ars-pr*::*mcherry*/*znt-pr*::*egfp* and *znt-pr*::*mcherry*/*ars-pr*::*egfp* were exposed to nine heavy metal and metalloid ions (Fig. 1). The induction coefficients of mCherry and eGFP were determined by fluorospectrometer from the dual-sensing WCBs exposed to 1 ppm (μg/mL) of As(III), As(V), Cd(II), Ni(II), Cu(II), Cr(VI), Zn(II), Pb(II), and Hg(II) for 2 h. The expression of the reporter genes in WCBs based on the promoter of metal inducible operon was induced by the association of metal ions with metal binding proteins, regulating transcription of the reporter genes. The WCB harboring *ars-pr*::*mCherry* and *znt-pr*::*eGFP* produced red and green fluorescence in response to arsenic and cadmium, respectively (Fig. 1a). When the sensing elements were switched each other to create the constructs *znt-pr*::*mCherry* and *ars-pr*::*eGFP*, the arsenic and cadmium induced eGFP and mCherry, respectively (Fig. 1b). Here, it was noticed that the metal selectivity was corresponded to the promoter region and its regulatory proteins. Although WCB based on *ars-pr* could not distinguish As(III) (arsenite) and As(V) (arsenate), it showed a stronger response to As(III), suggesting that the biological effects of As(III) to *E. coli* is stronger than that of As(V).

In addition, we further investigated the response of WCBs toward higher concentration of metal(loid)s ions to confirm the metal selectivity using the WCB harboring pET_ars-pr-mCherry/pCDFDuet_znt-pr-eGFP. When WCBs were exposed to 10 ppm (μg/mL) of metal(loid) ions for 1 and 2 h, the expression of mCherry corresponding to arsenic was induced only As(III), while eGFP corresponding cadmium was induced by Zn(II), and Cr(II) as well as Cd(II) (Fig. S1). Although Zn(II) and Cr(II) induced the expression of eGFP and the induction

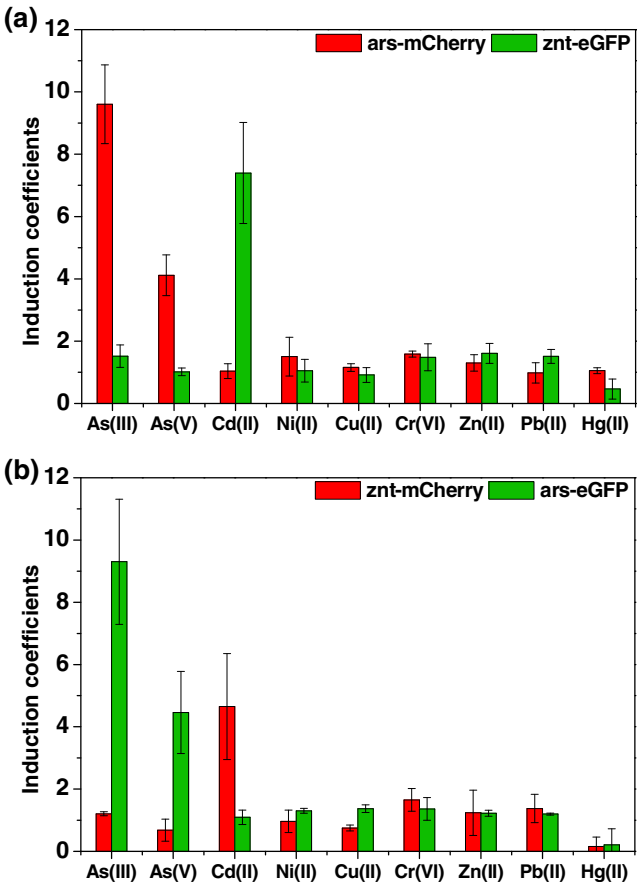

Fig. 1 The induction coefficients of mCherry and eGFP in dual-sensing WCBs exposed to 1 μg/mL of nine heavy metal(loid)s for 2-h exposure. **a** Induction coefficients of mCherry and eGFP in WCB harboring pCDFDuet_Znt-eGFP/pET_Ars-mCherry. **b** Induction coefficients of mCherry and eGFP in WCB harboring pET_Ars-eGFP/pCDFDuet_Znt-mCherry. The responses of the dual-sensing WCBs toward heavy metals are represented as the induction coefficients from three replicated tests. *Error bars* represent the standard deviation

coefficient increased with longer exposure duration (over 5 h), it was much less than that from Cd(II). In the strict sense of the word, cadmium sensing was not as specific as arsenic sensing. Nonetheless, since the dual-sensing WCB showed the expression of eGFP by only Cd(II) under this experimental condition, it would be used to quantify bioavailable arsenic and cadmium in contaminated soils.

Differential responses of the dual-sensing WCBs to As(III) and Cd(II)

Since heavy-metal selectivity was tested for each of the heavy metals separately, we verified the differential responses of the dual-sensing WCBs to combined treatment with As(III) and Cd(II) to exclude cross-effects. As shown in Fig. 2, the expression of reporter genes were induced by each metal differentially when both WCBs harboring *ars-pr*::*mCherry*/*znt-pr*-*eGFP* and *ars-pr*::*eGFP*/*znt-pr*::*mCherry* were exposed to

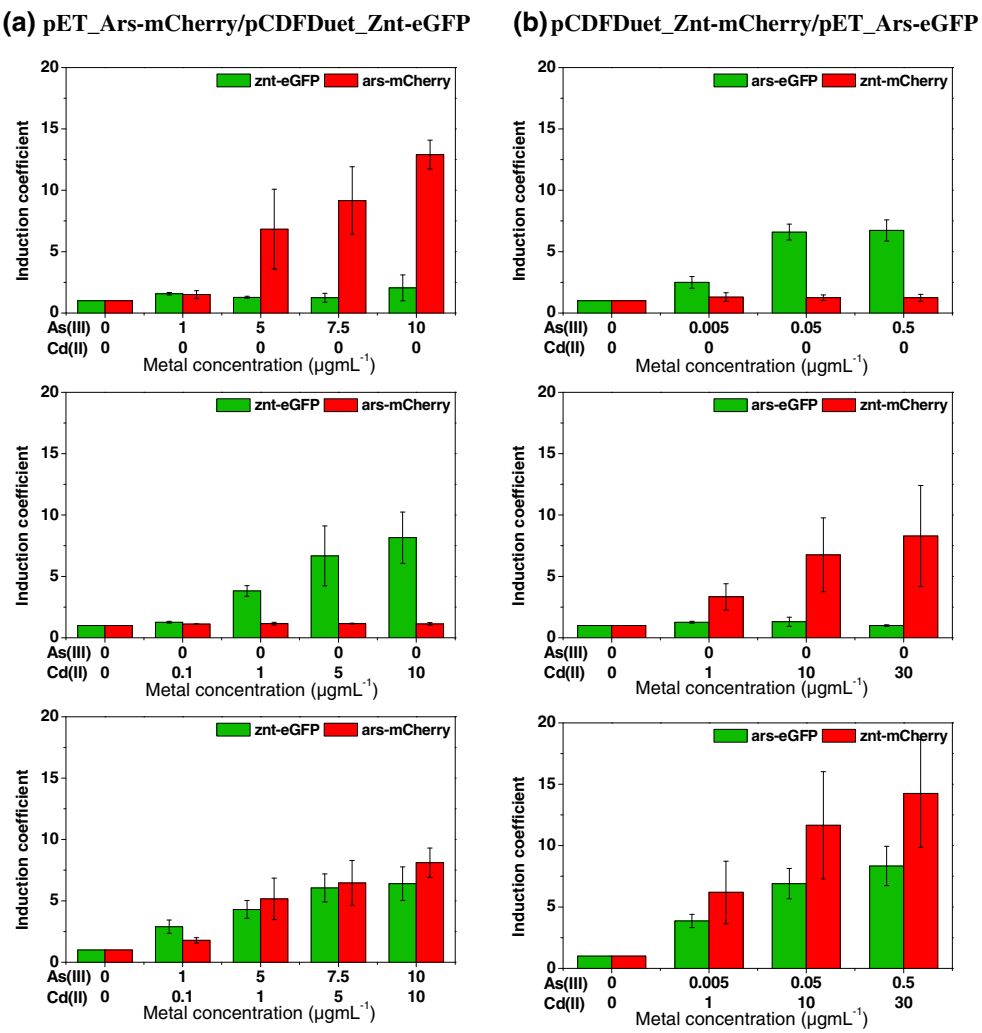

Fig. 2 Differential response toward As(III) and Cd(II) of WCBs harboring arsenic- and cadmium-sensing plasmids. **a** Induction coefficients of WCB harboring pCDFDuet_Znt-eGFP/pET_Ars-mCherry upon treatment with As(III) and Cd(II) alone and in combination. **b** Induction coefficients of WCB harboring pET_Ars-eGFP/pCDFDuet_Znt-mCherry. The induction coefficients of mCherry and eGFP are indicated as *red* and *green bars*, respectively. The induction of reporter genes was proportional to the concentration of heavy metals and the WCB responded differentially to As(III) and Cd(II). The induction coefficients were determined from at least three replicated tests. *Error bars* represent the standard deviation (color figure online)

comparative and combined As(III) and Cd(II). In case of comparative treatment, the expression of mCherry and eGFP was induced by their corresponding metal ions specific and the intensity signal was dose-dependent. In case of combined treatment, both mCherry and eGFP were induced and the induction coefficients were comparable to those from comparative treatment. This result confirmed that the dual-sensing WCBs specifically and differentially responded to both metals, indicating that they can effectively be applied for simultaneous monitoring of bioavailable arsenic and cadmium in the environment. Additionally, we further investigated the detection capability of single- and dual-sensing WCBs by comparing the induction coefficients at the same experimental conditions. The induction coefficient of mCherry and eGFP in both single- and dual-sensing WCBs were determined with same concentration of As(III) and Cd(II). Since the responses toward As(III) and Cd(II) of both WCBs were similar each other, it was concluded that the capability of dual-sensing WCBs to detect As(III) and Cd(II) was comparable to single-sensing WCBs (Fig. S2).

Fluorescence microscopic analysis of differential response of dual-sensing WCBs

In order to confirm the differential expression of reporter genes by its corresponding metal ions, the WCBs exposed to metal ions was analyzed by fluorescence microscopy. The fluorescent images of WCBs harboring *ars-pr*::*mCherry*/*znt-pr-eGFP* exposed to As(III) and Cd(II) comparatively and combined for 2 h are shown in Fig. 3. No signal was detected from the WCB cells without metal ions (Fig. 3a), and comparative treatment of As(III) and Cd(II) induced the differential expression of mCherry and eGFP, respectively (Figs. 3b, c). However, it was noticed that very few cells showed weak expression of the second reporter gene. This could be attributed by the basal level of expression or by nonspecific responses from opposite metal ions. In case of combined metal treatment, both reporter genes were strongly expressed in the WCB (Fig. 3d). This result corroborated our finding that the dual-sensing WCB responded differentially and specifically to As(III) and Cd(II).

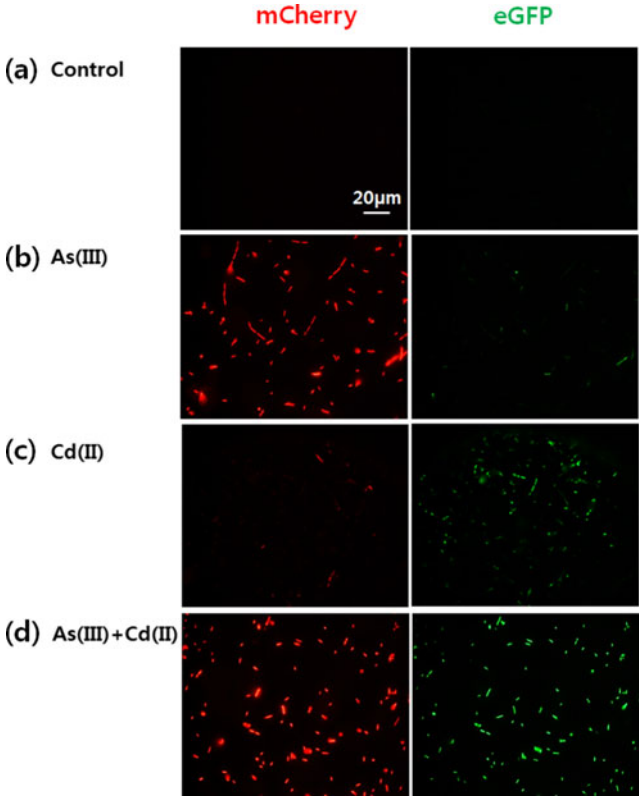

Fig. 3 Fluorescence images of *ars-pr*::*mCherry*/*znt-pr-eGFP* WCB exposed to As(III) and Cd(II) alone and in combination. The images were taken using a fluorescence microscope with 470/510 and 580/610 nm as excitation/emission wavelength filter sets for eGFP and mCherry, respectively, and ±25 nm of bandwidth. **a** Control, WCB without heavy metal exposure; **b** As(III), WCB exposed to 0.5 ppm (μg/mL) of As(III); **c** Cd(II), WCB exposed to 0.5 ppm of Cd(II); **d** As(III) + Cd(II), WCB exposed to 0.5 ppm of both As(III) and Cd(II)

Simultaneous detection of bioavailable As(III) and Cd(II) in amended LUFA soils

The capability of dual-sensing WCBs to quantify the bioavailable arsenic and cadmium was investigated in amended LUFA soils. Even if the heavy metals and metalloids are present as diverse chemical states, it is practically impossible to distinguish them by WCB assay. Hence, we assumed herein the bioavailable arsenic and cadmium was As(III) and Cd(II), and the standard curves were also obtained from the relationship between the induction coefficients and the concentrations of As(III) and Cd(II). Since the amount of bioavailable heavy metals in soils is generally much lower than the total amount, amended LUFA soil samples were contaminated with high concentrations of As(III)/Cd(II) (100/100, 100/50, and 200/10 μg/g). To quantify the bioavailable As(III) and Cd(II) in the amended LUFA soils, the induction coefficient of each test units containing 0–1 μg/mL of As(III) and Cd(II) ions in inversed order was plotted against the concentration. As shown in Fig. 4a, it was observed that the induction coefficient of each reporter gene was increased in the concentration-

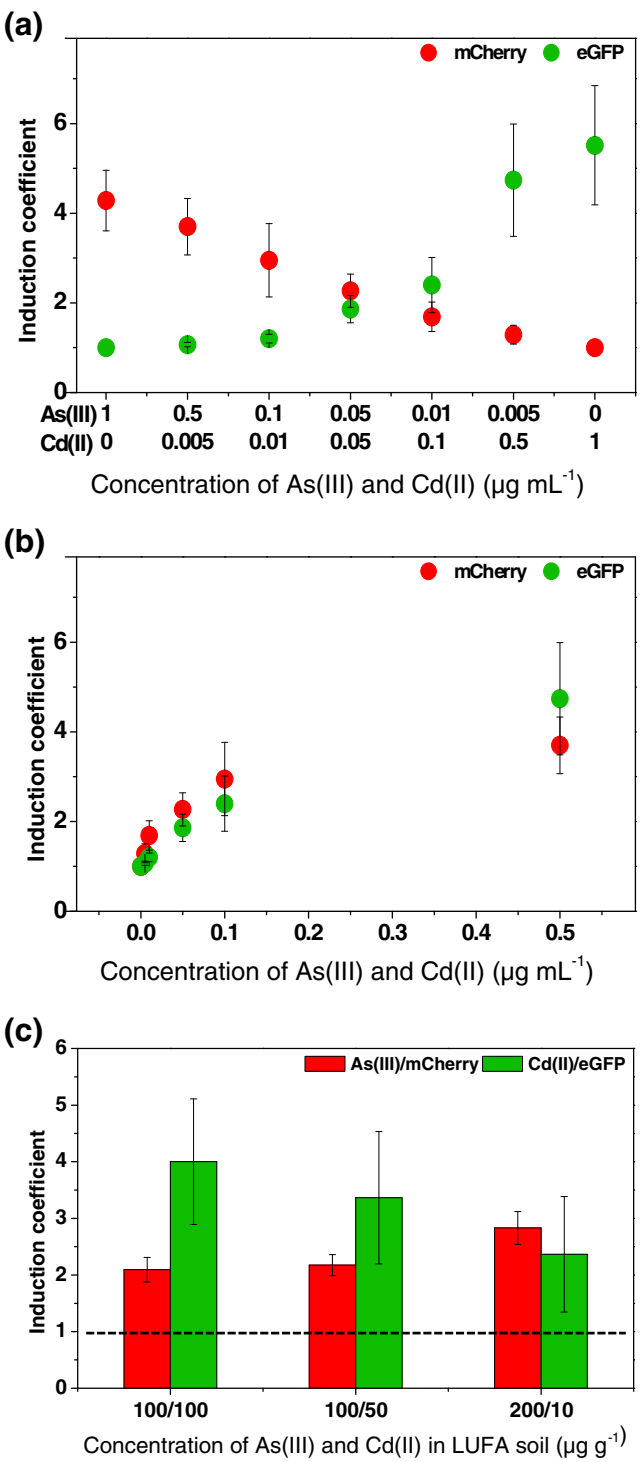

Fig. 4 Quantification of bioavailable As(III) and Cd(II) in amended LUFA soils using the *ars-pr*::*mCherry*/*znt-pr-eGFP* dual-sensing WCB. **a** Induction coefficients of mCherry and eGFP differentially induced by As(III) and Cd(II) in a concentration-dependent manner. **b** Standard curve for quantification of As(III) and Cd(II) in amended LUFA soils based on induction coefficients of mCherry and eGFP in 0–0.5 ppm of both metals. **c** Induction coefficients of both mCherry and eGFP in the WCB exposed to amended LUFA soils. The induction coefficients were determined from five replicated tests. *Error bars* represent the standard deviation

dependent manner, and the reporter genes were differentially expressed in accordance with the activated sensing elements. Each standard curve was obtained from the induction coefficients in the range of 0–0.5 μg/mL by using a single rectangular hyperbola regression (Fig. 4b). The induction coefficients of mCherry and eGFP from dual-sensing WCBs exposed to each amended LUFA soil sample was obtained by following the same procedure as described above and shown in Fig. 4c. The amount of bioavailable As(III) and Cd(II) in the amended LUFA soil was calculated and the concentration was expressed in micrograms per gram of soil (Table 1). The portion of bioavailable As(III) was 1.07, 1.27, and 1.65 % in 100/100, 100/50, and 200/10 of As(III)/Cd(II)-spiked samples, respectively. As shown in Table 1, the As(III)/Cd(II) samples at ratios of 100/50 and 100/100 showed a similar level of bioavailable As(III), corroborating that the dual-sensing WCB is specific and responds differentially to each heavy metal ion. The amount of bioavailable Cd(II) increased with increasing spiked Cd(II) concentration regardless of the arsenic amount, again supporting the differential responding nature of the dual-sensing WCB to arsenic and cadmium ions.

Dual quantification of bioavailable arsenic and cadmium in contaminated site soils

Although we showed that the dual-sensing WCBs are able to simultaneously quantify arsenic and cadmium in amended LUFA soils, the tested concentrations of arsenic and cadmium were not relevant with regard to environmental systems. To test the applicability on environmental samples, the contaminated site soils were analyzed by the dual-sensing WCB assay. The site soils were collected from six locations around a smelter area in Korea. To exclude the effects of other heavy metals, the concentrations of arsenic, cadmium, copper, nickel, lead, and zinc in the site soils were determined by ICP-AES as 23.3 (9.6–46.4), 1.8 (0.21–3.37), 61.5 (17.8–89.9), 12.5 (9.1–22.0), 96.0 (35.3–158.6), and 128.8 (50.6–252.9) μg/g, respectively. Although the contaminated site soils contained high concentrations of other heavy metals such as copper, lead, and zinc, these can be neglected because the soil samples were diluted 50 times in the test units and the bioavailable portion was much less than the total amount. Moreover, it was previously verified that the dual-sensing WCB did not respond to these metals at 1 μg/mL with 2-h exposure (Fig. 1).

To quantify the bioavailable As(III) and Cd(II) in the contaminated site soils, the standard curves were constructed from the test units containing 0–0.5 μg/mL of metal ions with LUFA soils as a reference. Strictly speaking, As(III) and Cd(II) as well as LUFA soil are no actual references for the WCB assay because heavy metals are present in diverse chemical states in soils and the physicochemical property of each site soil is different. However, to allow interlaboratory standardization of the assay and comparison of results, we used the LUFA soils as references.

Table 1 The amount of total and bioavailable As(III) and Cd(II) in amended LUFA soils and contaminated site soils determined by ICP-AES analysis and dual WCB assay, respectively

Soil samples		Arsenic			Cadmium		
		Total (ICP-AES)	Bioavailable (WCB assay)	Bioavailability (%)[a]	Total (ICP-AES)	Bioavailable (WCB assay)	Bioavailability (%)[a]
Amended soils	100/100	100	1.07 ± 0.112	1.07	100	15.62 ± 0.36	15.62
	100/50	100	1.27 ± 0.108	1.27	50	10.13 ± 0.52	20.26
	200/10	200	3.29 ± 0.298	1.65	10	4.61 ± 0.13	46.10
Contaminated sites soils	Site 1	21.62 ± 2.12	0.23 ± 0.054	1.04	2.04 ± 0.096	ND	–
	Site 2	46.43 ± 3.52	0.17 ± 0.033	0.37	3.32 ± 0.057	ND	–
	Site 3	12.89 ± 0.56	0.35 ± 0.095	2.72	1.80 ± 0.072	0.06 ± 0.049	3.33
	Site 4	17.35 ± 2.34	0.32 ± 0.027	1.84	2.40 ± 0.086	0.15 ± 0.027	6.38
	Site 5	32.65 ± 0.98	0.19 ± 0.077	0.58	0.25 ± 0.837	0.02 ± 0.032	7.29
	Site 6	9.54 ± 1.12	0.31 ± 0.060	3.24	0.95 ± 1.038	0.06 ± 0.030	6.72

The amount of heavy metals determined by ICP-AES and WCB assay were expressed per gram of soil; the unit of concentration of arsenic and cadmium was ppm ($\mu g\ g^{-1}$); the total amount of arsenic and cadmium in amended soil was the concentration spiked into the LUFA soils

ND the bioavailable cadmium in sites 1 and 2 was too low to be determined by WCB assay

[a] Bioavailability represents the portion of bioavailable fraction to total metal contents, calculated as [bioavailable] / [total] × 100

The relationship between the induction coefficient of each reporter and the concentration of arsenic and cadmium was analyzed using nonlinear regression, yielding R^2 values of 0.9980 and 0.9987 for arsenic and cadmium, respectively (Fig. 5a). The amounts of bioavailable arsenic and cadmium in the site soils were calculated from the induction coefficients of the WCB exposed to each sample (Fig. 5b), and are listed in Table 1. The induction coefficient of mCherry ranged between 1.58 and 2.03, corresponding to 0.17–0.35 μg of bioavailable arsenic per 1 g of soil. In contrast, the induction coefficients of eGFP was slightly over 1, indicating very low level of bioavailable cadmium was detected from the contaminated soils. However, the portion of bioavailable cadmium (0–7.29 %) was higher than arsenic (0.58–3.24 %) that was as same trend as observed from amended contaminated soil test. Thus, it was proposed that cadmium remains more as biologically active forms than arsenic in soils. This was reasoned by the nature of arsenic and cadmium in soil was different and suggesting arsenic associated tighter with soils than cadmium. However, the physicochemical properties of both metals and soils, which affect the formation of diverse heavy metal species, should be taken into consideration. In this respect, risk assessment based on the total amount of metals in a soil by using analytical instruments would not be accurate. Thus, quantitative measurement of metal bioavailability using WCBs would be invaluable to achieve appropriate evaluation of heavy metal toxicity.

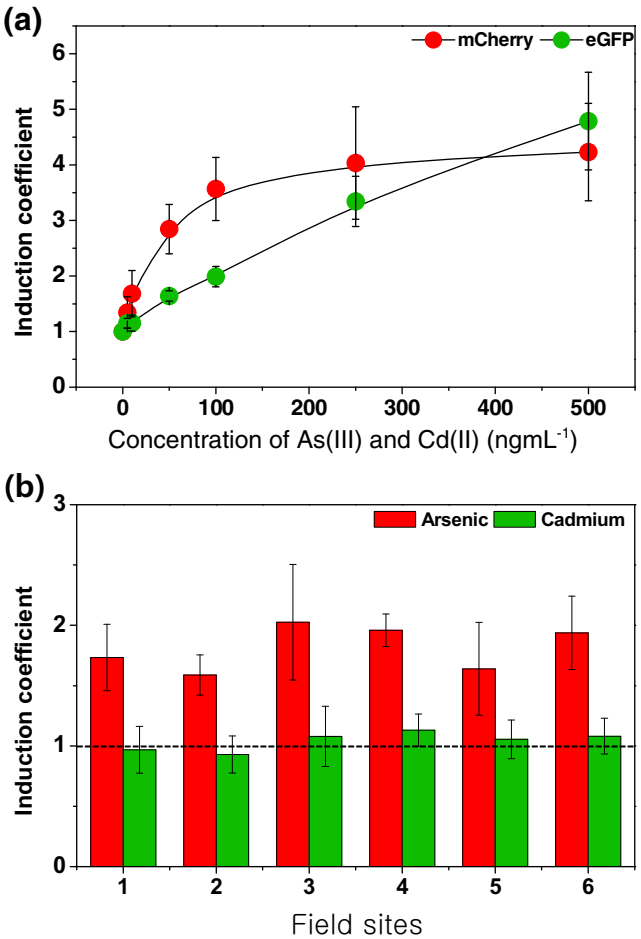

Fig. 5 Quantification of bioavailable arsenic and cadmium in contaminated field soils using the *ars-pr*::*mCherry*/*znt-pr-eGFP* dual-sensing WCB. **a** Standard curves for quantification were obtained by plotting the induction coefficients of mCherry and eGFP against the concentration of As(III) and Cd(II). **b** Induction coefficients of mCherry and eGFP in the WCB indicating bioavailable arsenic and cadmium, respectively. The values represent the average of five replicated tests. *Error bars* represent standard deviation. The *dotted line* indicates the induction coefficients of the WCB not exposed to a metal

Discussion

Many whole-cell bacterial bioreporters are current available to monitor bioavailable amount of pollutants in diverse environmental systems. Most remarkable feature of WCBs would be the ability to measure not total amount but the bioavailability of pollutants which provides more information for assessing the risk; thereby, it was actively developed during past few decades. Currently available WCBs were varied by the target pollutants such as heavy metals, aromatic hydrocarbons, DNA damaging agents and antibiotics, and species of bacterial strains including *E. coli*, *Pseudomonas putida*, *Lactococcus lactis*, and *Bacillus subtilis* (Robbens et al. 2010; van der Meer and Belkin 2010). However, all of them share similar mechanism based on the fusion of the promoters of stimuli-inducible genes with reporter genes to detect bioavailability of pollutants. Thus, the selectivity and sensitivity of WCBs is determined by the properties of sensing elements, reporter genes, and host bacterial strains.

Among the diverse environmental pollutants, the WCBs to detect the heavy metals and metalloids in environments such as cadmium, lead, mercury, lead, and arsenic were developed mostly because of the severe adverse effects of heavy metals on human (Jain and Ali 2000; Nachman et al. 2005; Tao et al. 2013; Wongsasuluk et al. 2014). As mentioned above, they are all employed the gene promoters in metal-inducible operons as sensing elements. As an example, the bioavailable arsenic was detected by WCBs employed the promoters in *ars* operon, and bioavailable cadmium was detected by using *cad* or *znt* operon (Kaur et al. 2015; Riether et al. 2001). However, currently, no study on simultaneous analysis of arsenic and cadmium has been reported, even though soils are rarely contaminated with one heavy metal alone. Thus, we demonstrated herein the development and application of dual-sensing WCBs to detect bioavailable arsenic and cadmium. Dual-color WCBs have been reported previously, but these did not specifically target two pollutants. In dual-color WCBs, one color generally indicates the amount of a specific pollutant similar to single-color WCBs, while the second color indicates the viability of bioreporter itself, rather than the amount of a second pollutant (Mirasoli et al. 2002; Wood and Gruber 1996). Alternatively, both colors can indicate broad biological

effects of combined rather than specific toxic materials (Belkin 2003). In contrast, the WCBs established in this study are arsenic and cadmium specific dual-sensing WCBs.

In present study, the dual-sensing WCBs were generated by using two different sets of metal-sensing elements with the reporter genes; *ars-pr*::*mCherry*/*znt-pr-eGFP* or *ars-pr*::*eGFP*/*znt-pr*::*mCherry*. The detection thresholds of dual-sensing WCBs for cadmium and arsenic calculated from the average and standard deviation of fluorescent intensity of blank samples following the reported method (Baumann and van der Meer 2007) were 3.6 ng/mL and 2.79 ng/mL, respectively, and these values were comparable to the detection thresholds of single-color WCBs ranged from 1 ng/mL to 5 ng/mL in the previous reports (Diesel et al. 2009; Magrisso et al. 2008; Robbens et al. 2010; van der Meer and Belkin 2010). Moreover, since the responses of dual-sensing WCBs to arsenic and cadmium were differentially induced without cross-selectivity effects, each heavy metal could be quantified using the WCBs. Based on the WCB assay, the amount of bioavailable arsenic and cadmium in soils was much higher than soil solutions consistent with the previous studies (Ivask et al. 2004; Maderova and Paton 2013; Song et al. 2014; Turpeinen et al. 2003; Yoon et al. 2015), and the difference between soil and soil solution (water extracts) was reported as 20- to 115-fold. However, there is no clear explanation why the bioavailable portion was higher in soil than the soil solution. It might be reasoned by extraction efficiency of metal ions from soil particles because there is no way to extract metal ions completely from soils, or by the changes of chemical states of metal ions. The latter was supported by the biomobilization of metals associated with soil particles to bacterial cells that was reported the previously (Petanen and Romantschuk 2003). Thus, we speculated that the metals associated with soil particles were transformed to biologically active forms (released ions from soil particles) by the contacts with bacterial cells. This process might happen between bacterial cell membrane and soil particles or by the dissociation of metal ions from soil particles inside bacterial cells even if it was not clarified yet.

Nonetheless, this is the first report of development of dual-labeled WCBs for simultaneous detection of two specific toxic elements to the best of our knowledge. Generally, soils contain multiple heavy metals rather than a single metal. The WCB established in this study allowed concurrent quantification of bioavailable arsenic and cadmium; therefore, it is time- and cost-effective. Although we only applied the dual-sensing WCB to contaminated soils, it is applicable not only to other environmental systems but also to diverse industrial fields requiring rapid and cost-effective quantification of bioavailable heavy metals. Furthermore, we presented a proof of concept for the development of WCBs that can detect multiple toxic materials by combining pollutant-specific sensing elements. Because a variety of reporter genes ranging from fluorescent to enzymatic proteins are available, multiple sets of engineered genes inserted into bacterial cells can act as different sensors if specific sensing elements are available. Therefore, we believe that the development of WCBs sensing multiple pollutants is feasible, and that multiple-sensing bioreporters would be efficient and useful tools to assess the risks of multiple-contaminants co-present in the environmental systems.

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Compliance with ethical standards

Ethical statement All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

Conflict of interest Youngdae Yoon declares that he has no conflict of interest. Sunghoon Kim declares that he has no conflict of interest. Yooeun Chae declares that she has no conflict of interest. Shin Woong Kim declares that he has no conflict of interest. Yerin Kang declares that she has no conflict of interest. Seung-Woo Jeong declares that he has no conflict of interest. Youn-Joo An declares that she has no conflict of interest.

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