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Functional characterization of Gram-negative bacteria from different genera as multiplex cadmium biosensors

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ABSTRACT

Widespread presence of cadmium in soil and water systems is a consequence of industrial and agricultural processes. Subsequent accumulation of cadmium in food and drinking water can result in accidental consumption of dangerous concentrations. As such, cadmium environmental contamination poses a significant threat to human health. Development of microbial biosensors, as a novel alternative method for *in situ* cadmium detection, may reduce human exposure by complementing traditional analytical methods. In this study, a multiplex cadmium biosensing construct was assembled by cloning a single-output cadmium biosensor element, *cadRgfp*, and a constitutively expressed *mrfp1* onto a broad-host range vector. Incorporation of the duplex fluorescent output [green and red fluorescence proteins] allowed measurement of biosensor functionality and viability. The biosensor construct was tested in several Gram-negative bacteria including *Pseudomonas*, *Shewanella* and *Enterobacter*. The multiplex cadmium biosensors were responsive to cadmium concentrations ranging from 0.01 to 10 μg ml^{-1}, as well as several other heavy metals, including arsenic, mercury and lead at similar concentrations. The biosensors were also responsive within 20–40 min following exposure to 3 μg ml^{-1} cadmium. This study highlights the importance of testing biosensor constructs, developed using synthetic biology principles, in different bacterial genera.

1. Introduction

Cadmium (Cd) is a naturally existing heavy metal found in Earth's crust at concentrations between 0.1–0.5 ppm. Industrial and agricultural processes (e.g. mining, battery manufacturing and use of phosphate fertilizers) has resulted in its widespread distribution in the environment (WHO, 2010). In some instances the release of Cd in soil and water has resulted in contamination of potable water (WHO, 2011) and food sources, such as grains (Ran et al., 2016; Simmons et al., 2005), fish (Canli and Atli, 2003) and meat (Jorhem et al., 1991). Ingestion of Cd contaminated substances may affect major organs as *in vivo* murine studies have observed damage and stress responses in the brain, kidneys, liver, spleen and testes (Agnihotri et al., 2015; Saygi et al., 1991; Thijssen et al., 2007). As such, decreasing exposure of human populations to Cd may mitigate potential health problems.

Analytical methods currently used for Cd detection, such as atomic absorption spectroscopy, are sensitive, time-consuming and costly. Microbial biosensors have the potential to complement existing analytical methods, allowing a preliminary *in situ* analysis of water and soil to be performed in real-time. Synthetic biology principles are key to the construction of genetic constructs whereby the expression of a reporter gene is linked to the presence of Cd in the cell's environment (Bereza-Malcolm et al., 2015). Previously developed Cd biosensor constructs generally incorporate genetic elements (*cadC*, *cadR* or *zntA*) from identified heavy metal resistance operons (Brocklehurst et al., 1999; Endo and Silver, 1995; Lee et al., 2001). The resistance mechanisms are based on the regulated expression of P-type ATPases or chemiosmotic cation/proton antiporters, which exude Cd from the cell (Silver, 1996). The Cd resistance operon is regulated by a transcriptional regulator which binds to a divergent operator/promoter region in the absence of Cd ions. For example, the *cadCA* genes on the pI258 plasmid of *Staphylococcus aureus* encode a regulatory protein (CadC) and an efflux pump (CadA), respectively (Endo and Silver, 1995; Smith and Novick, 1972). Cd resistance genes have also been identified in Gram-negative bacteria, such as *czcABC*, on the pMOL30 plasmid of *Alcaligenes eutophus* CH34 (Nies, 1995), *zntA* on the chromosome of *Escherichia coli* (Brocklehurst et al., 1999) and *cadR* in *Pseudomonas putida* 06909 (Lee et al., 2001). The majority of existing

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Cd biosensors are 'single-output' biosensors, where the presence of Cd results in the production of a specific output, including bioluminescence, fluorescence or pigment production (Joe et al., 2012; Tao et al., 2013; Tauriainen et al., 1998). While useful for gaining an understanding of the regulatory genes used, limited information is provided on the metabolic health of the bacterium. Development of multiplex microbial biosensors could provide more reliable outputs to monitor the metabolic state of the cell and variability induced by environmental fluctuations. Additionally, while several bacterial species have been utilized as Cd biosensors, including *E. coli* (Biran et al., 2000), *Pseudomonas fluorescens* (Ivask et al., 2009), *P. putida* (Wu et al., 2009), *S. aureus* and *B. subtilus* (Tauriainen et al., 1998), no study has yet analysed the same construct in different species subjected to the near identical conditions. Observed differences in detection limits and lack of specificity between previously developed Cd biosensors are generally associated with the regulatory gene used. We speculated that the bacterial species expressing the biosensor construct may be contributing to the observed differences (Bereza-Malcolm et al., 2015). Subsequently, this paper aims to address several areas of microbial biosensor development and analysis which have been underrepresented.

In this study a multiplex Cd biosensor was developed for use in Gram-negative bacteria. A single-output Cd biosensor construct (Tao et al., 2013), containing *cadR* and its native divergent operator/promoter, P_{cadR} and P_{cad}, from the chromosome of *P. putida* 06909 (Lee et al., 2001), upstream of a promoterless *gfp* was cloned into a low-copy number, broad host range plasmid, pBBR1MCS-5 (Kovach et al., 1995). A constitutively expressed *mrfp1* was also cloned into the same construct. As such, the output consisted of a duplex fluorescent output [green and red fluorescent protein (GFP and RFP, respectively)] where the presence of Cd results in GFP expression. The constitutive expression of RFP and measurement of bacterial growth ($OD_{600\ nm}$) allowed assessment of biosensor functionality and viability. The biosensor construct was transferred into *Pseudomonas aeruginosa* PAO1 and *Shewanella oneidensis* MR-1, as well as two *Enterobacter* spp. (designated NCR3 and LCR17) which were isolated from Cd-contaminated soil (Egidi et al., 2016; Liu et al., 2015). These genera were chosen because of their widespread distribution in water and soil, as well as their roles in bioremediation (Liu et al., 2015; Selezska et al., 2012; Tiedje, 2002; Viamajala et al., 2002). The biosensors presented in this study were tested through a series of qualitative and quantitative analyses, based on a set of guidelines previously proposed (Bereza-Malcolm et al., 2015). To the best of authors' knowledge, this is the first report of a multiplex Cd biosensor construct which has been tested in several Gram-negative bacteria.

2. Materials and methods

2.1. Reagents

Stock solutions at 10^4 μg ml^{-1} of antimicrobial agents (antibiotics and heavy metals) and diaminopimelic acid (DAP) were prepared by dissolving 0.1 g in 10 ml of dH_2O, excluding chloramphenicol which was dissolved in methanol. Heavy metals (i.e. sodium arsenite [As(III)], cadmium chloride [Cd(II)], copper chloride [Cu(II)], chromium oxide [Cr(VI)], lead nitrate [Pb(II)], mercury chloride [Hg(II)] and zinc chloride [Zn(II)]) used in fluorescence assays were stored at room temperature and diluted as necessary. Antibiotics and DAP stock solutions were stored at −20 °C, and thawed prior to use. All antimicrobial agents and DAP were obtained from Sigma-Aldrich Pty Ltd.

2.2. Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S1. *E. coli* DH5α, *E. coli* WM3064 and *P. aeruginosa* PAO1 were grown at 37 °C, while *S. oneidensis* MR-1, *Enterobacter* spp. NCR3 and LCR17 were grown at 28 °C. Bacterial strains were maintained on nutrient agar (NA) [3.5% blood agar base (w/v); 1.5% (w/v) agar, 1% (w/v) Lab-Lemco powder, 1% (w/v) peptone, 0.5% (w/v) sodium chloride and 0.5% (w/v) yeast extract] and in nutrient yeast broth (NYB) [2.5% (w/v) nutrient broth and 0.5% (w/v) yeast extract]. Antimicrobial agents were added to NA and NYB, as necessary. The pBBR1MCS-5, pSB1C3 and pPROBE-NT plasmids were selected for using 10 μg ml^{-1} gentamicin sulphate, chloramphenicol or kanamycin sulphate, respectively. For culturing the auxotrophic *E. coli* WM3064, NA and NYB were supplemented with 10 μg ml^{-1} DAP.

2.3. Construction of the cadmium biosensor constructs

The genetic element of the single-output Cd biosensor construct consists of *cadR* (444 bp) and the divergent operator/promoter region (P_{cadR} and P_{cad}; 84 bp) that it regulates from the chromosome of *Pseudomonas putida* 06909 (NCBI Accession no. AF333961; 528 bp), and a promoterless *gfp* gene from pPROBE-NT (*gfp*)(717 bp). The Cd biosensor construct (pPROBE-NT*cadRgfp*) was gifted from Tao et al. (2013), and the genetic element, *cadRgfp* (1301 bp), was PCR amplified. To allow subsequent cloning of the genetic element, *Hin*dIII restriction sites were incorporated into the forward (5′- CCC AAG CTT TTA ATG CCC GTG GCT TCG CCC TAC AT-′3) and reverse (5′-ATT ACT AGT AAG CTT CTA TTT GTA TAG TTC ATC CA-3′) primers (Integrated DNA Technologies Pty Ltd).

The PCR product was gel purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) prior to digestion with *HindIII* and cloning into the low-copy number, broad host range vector, pBBR1MCS-5 (4768 bp), producing the single-output construct (pBB*cadRgfp*; 6069 bp). To construct the multiplex Cd biosensor, *mrfp1* under the transcriptional control from P_{lacI} (BBa_J04450; 1069 bp) was digested from pSB*rfp* using *Xba*I and *Spe*I and subcloned, using the same restriction sites, into the single-output Cd construct, pBB*cadRgfp* (producing pBB*cadRgfp-rfp*; 7144 bp). The resulting construct was screened through plasmid extraction and restriction digestion at 37 °C for 1 h followed by gel electrophoresis to visualise the release of the correct sized fragment prior to confirmation via Sanger sequencing (AGRF) and sequence alignment to the predicted construct. A plasmid map of pBB*cadRgfp-rfp* was generated using Snapgene® software (GSL Biotech) (Fig. S1). For comparative purposes, *gfp* and *mrfp1* under constitutive expression (from P_{tetR} and P_{lacI}, respectively) were subcloned into pBBR1MCS-5; denoted pBB*gfp* and pBB*rfp*. A nalidixic acid resistant (7 μg ml^{-1}) laboratory strain *E. coli* DH5α was used for assembly of the aforementioned constructs.

2.4. Conjugation procedure

All constructs were transferred via electroporation into auxotrophic *E. coli* WM3064 to allow conjugal transfer of the plasmids. The exponential phase donor [*E. coli* WM3064 containing either (pBB*cadRgfp-rfp*), (pBB*cadRgfp*), (pBB*gfp*) or (pBB*rfp*)] and recipient (either *P. aeruginosa* PAO1, *S. oneidensis* MR-1, *Enterobacter* spp. NCR3 and LCR17) NYB-cultures were (50 μl each) spotted onto a pre-warmed NA plate. The plate was placed under condition suitable for optimal growth of the recipient (37 °C for *P. aeruginosa* PAO1 or 28 °C for *S. oneidensis* MR-1 and *Enterobacter* spp.). Following a 4-h incubation, the bacteria were scraped from the NA and streaked onto fresh NA supplemented with 10 μg ml^{-1} gentamicin sulphate and incubated under appropriate conditions. Donor and recipient cultures were plated separately onto the same selective medium to serve as negative controls.

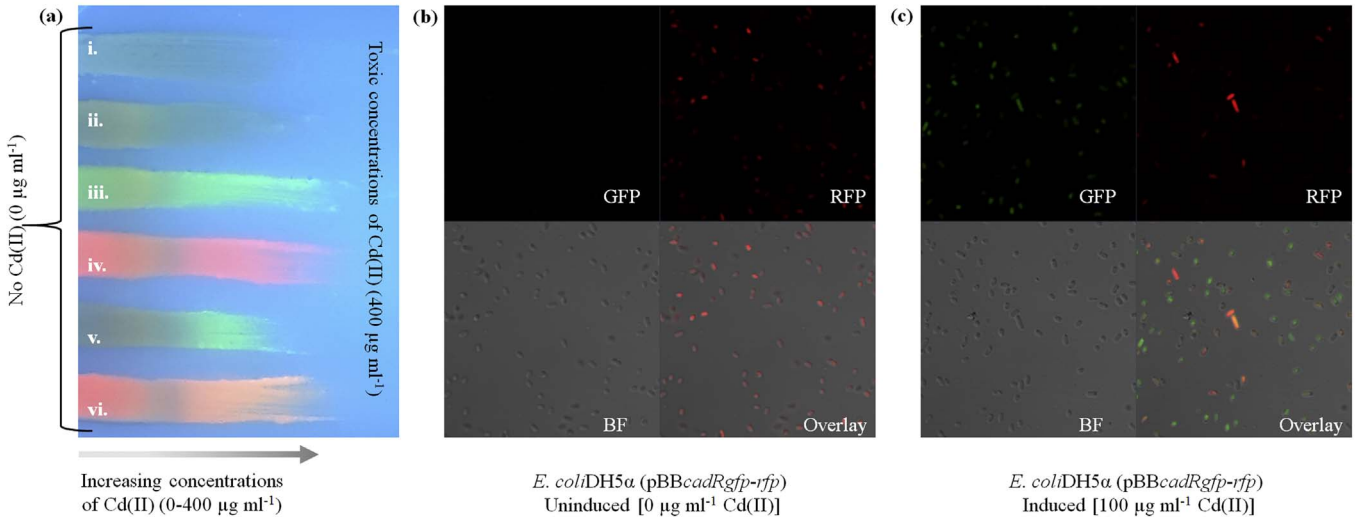

Fig. 1. Qualitative analyses of DH5α(pBB*cadRgfp-rfp*) biosensor using slope ager plates and via fluorescence microscopy **(a)** Slope agar plates; Cd biosensors and other constructs were streaked over a Cd(II) gradient in the following order; (i) DH5α (ii) DH5α(pBB) (empty vector); (iii) DH5α(pBB*gfp*) (constitutively expressed GFP); (iv) DH5α(pBB*rfp*) (constitutively expressed RFP); (v) DH5α(pBB*cadRgfp*) (single-output Cd biosensor) and (vi) DH5α(pBB*cadRgfp-rfp*) (multiplex Cd biosensor). Fluorescence microscopy; uninduced cells **(b)** and cells induced with 100 μg ml^{-1} Cd(II) **(c)** were viewed under brightfield (BF); green fluorescence filter (GFP); red fluorescence filter (RFP); and an overlay of the two images, at ×1000.

2.5. Fluorescence microscopy

Early or mid-exponential phase ($OD_{600\ nm}$=0.2–0.6) NYB-cultures of multiplex Cd biosensors containing 10 μg ml^{-1} gentamicin sulphate were induced with 100 μg ml^{-1} Cd(II) for 4 h to allow expression of GFP. The uninduced NYB-culture served as a negative control. After 4 h, cells were washed three times in dH_2O to reduce refraction and resuspended in 100 μl dH_2O. A drop of culture (10 μl) was placed onto a glass slide and covered with a cover slip. The cover slip was firmly pressed onto the slide and sealed using nail polish. Samples were viewed using a Zeiss 510 confocal microscope. Brightfield and the RFP filter (excitation 544 nm and emission 610 nm) were used to view all the bacterial cells, the GFP filter (excitation 485 nm and emission 535 nm) was used to visualise the Cd- 'responsive" cells at ×1000 magnification with oil immersion.

2.6. Slope agar plates

Slope agar plates were assembled using NA to form a Cd(II) concentration gradient ranging from no Cd(II) (0 μg ml^{-1}) to inhibitory concentrations (400 μg ml^{-1}). (See supplemental materials, Section S1.1, Fig. S2). The comparative constructs and Cd biosensors [*E. coli* DH5α and *E. coli* DH5α containing (pBB), (pBB*gfp*), (pBB*rfp*), (pBB*cadRgfp*) or (pBB*cadRgfp-rfp*)] were streaked parallel to the concentration gradient and incubated overnight in the dark. Plates were visualized using a UVP ultraviolet transilluminator and photographed.

2.7. Fluorescence assays

Overnight NYB-cultures (10 ml) of the multiplex Cd biosensors were subcultured and incubated at appropriate temperatures for ~3 h (Table S2). This was the predetermined optimal time for all bacterial species to reach early or mid-exponential phase ($OD_{600\ nm}$=0.2–0.6). The exponential-phase NYB-cultures (900 μl) were exposed to varying concentrations of heavy metals (0.01–1000 μg ml^{-1}) for up to 4 h in microcentrifuge tubes (1 ml final volume). For environmental assays, exponential phase NYB-cultures (10 ml) were concentrated by centrifuged and resuspended in fresh NYB (1 ml). Resuspended cells (100 μl) were added to 800 μl of either ocean water (Bass strait, Australia), groundwater (Victoria, Australia) or dH_2O and mixed with 100 μl of varying concentrations of Cd(II) (0–5 μg ml^{-1}). Identical cultures were exposed to dH_2O to serve as a comparison to the environmental samples. Cultures were incubated in the dark with shaking for up to 4 h. After three cell washes with dH_2O, cells were aliquoted (200 μl) into black 96-well plates with clear flat bottoms and the relative fluorescence units (RFU) measured using a Clariostar plate reader. The excitation and emission values for GFP and RFP were 485 nm and 535 nm, and 544 nm and 610 nm, respectively. The average RFU of triplicate wells was calculated along with the average $OD_{600\ nm}$. To calculate the induction coefficient (IC), the calculated RFU per $OD_{600\ nm}$ for every data point was divided by the uninduced sample (in each experiment the uninduced sample's IC is 1). Significance was determined from RFU or RFU per $OD_{600\ nm}$ through the use of the students T-test ($P < 0.05$). A minimum of three biological replicates were performed for each assay.

3. Results and discussion

3.1. Qualitative analyses of the multiplex cadmium biosensors revealed differences in fluorescence intensity between bacterial species

E. coli DH5α was used as the host organism for qualitative assessment of the functionality of the multiplex Cd biosensor construct (pBB*cadRgfp-rfp*). Slope agar plates and fluorescence microscopy were used as preliminary tests as they are able to report viability of the bacterium via RFP expression and presence of Cd(II) via GFP expression. Slope agar plates contained an increasingly toxic Cd(II) concentration gradient (ranging from 0 to 400 μg ml^{-1}) (Fig. S2). The single [DH5α(pBB*cadRgfp*)] and multiplex-output [DH5α(pBB*cadRgfp-rfp*)] biosensors were streaked along the concentration gradient together with DH5α, DH5α(pBB), DH5α(pBB*gfp*) and DH5α(pBB*rfp*) (Fig. 1a). DH5α and DH5α(pBB) did not produce any visible fluorescence (Fig. 1a; i–ii), while DH5α containing either pBB*gfp* or pBB*rfp* expressed GFP and RFP, respectively (Fig. 1a; iii–iv). In the absence of Cd(II), there was no visible fluorescence from DH5α(pBB*cadRgfp*). However, upon exposure to Cd(II), GFP expression increased corresponding to increasing Cd(II) concentrations (Fig. 1a; v). A similar pattern of fluorescence was observed from DH5α(pBB*cadRgfp-rfp*) with RFP expression being present in the absence of Cd(II) followed by an increasing fluorescent yellow pigmentation (Fig. 1a; vi). This indicates that GFP and RFP are expressed concurrently as the biosensor grows and the multiplex Cd biosensor construct functions

as predicted. Both Cd biosensors and the comparative constructs had their growth inhibited upon reaching toxic concentrations of Cd(II). The darker band visible throughout the plate is resultant from the construction method of the slope agar plates and did not affect qualitative assessment. The use of slope agar plates provides a simple, useful and relatively rapid method for visualization of gene expression induced in the presence of antimicrobial agents while utilizing minimal equipment. As such, this method may be applied to other applications outside of biosensor development.

Biosensor functionality was further confirmed by fluorescence microscopy. An exponential phase NYB-culture of DH5α(pBBc*adRgfp-rfp*) was viewed under the fluorescence microscope following incubation for 4 h in the presence of Cd(II) (100 μg ml^{-1}). An identical NYB-culture lacking Cd(II) served as the negative control (Fig. 1b). Green fluorescent cells were only observed in the induced culture, confirming biosensor functionality (Fig. 1c). Expression of RFP was observed among the majority of cells from both the uninduced and induced NYB-cultures. Differences in GFP and RFP intensity among individual cells of the same culture were noted and are an indication of the variability within any given culture.

3.2. Analyses of multiple outputs revealed differences in biosensor functionality

Initially, the five multiplex Cd biosensors [DH5α(pBBc*adRgfp-rfp*), PAO1(pBBc*adRgfp-rfp*), MR-1(pBBc*adRgfp-rfp*), NCR3(pBBc*adRgfp-rfp*) and LCR17(pBBc*adRgfp-rfp*)] were exposed to a range of Cd(II) concentrations (0–1000 μg ml^{-1}) to assess their detection range and the effect of varying Cd(II) concentrations on growth ($OD_{600\ nm}$) and RFP expression (Fig. 2). Upon exposure to 0.01–100 μg ml^{-1} Cd(II), the induction coefficient (IC) of all five multiplex Cd biosensors increased (Fig. 2a) but decreased at higher concentrations (> 400 μg ml^{-1}), presumably due to the toxicity of Cd(II) inhibiting cell growth. There were significant differences in IC between the multiplex Cd biosensors although conditions were similar. MR-1(pBBc*adRgfp-rfp*) had the lowest IC (1.8 ± 0.14 at 10 μg ml^{-1}) while NCR3(pBBc*adRgfp-rfp*) (4.6 ± 0.32 at 10 μg ml^{-1}). PAO1(pBBc*adRgfp-rfp*) had the second lowest IC after MR-1(pBBc*adRgfp-rfp*), although it tolerated higher concentrations of Cd(II) with the highest IC (3.95 ± 0.19) of all the biosensors at 800 μg ml^{-1}. The differences in IC between bacterial species maintaining the same biosensor construct may be accounted for by differences in growth conditions and growth rates. Studies have shown that optimizing temperature and growth conditions (i.e. growth medium) for different bacterial species can alter detection limit and consequently the IC (Tao et al., 2013; Tauriainen et al., 1998). However, the observation of significant differences in the IC when comparing multiplex Cd biosensors under the same conditions may also be explained by differences in transcription rates, which are expected to differ between bacterial species encoding foreign DNA.

Previously developed Cd(II) biosensors rely on a single-output (Ivask et al., 2009, 2002; Joe et al., 2012; Tao et al., 2013; Tauriainen et al., 1998). In this study, an additional fluorescent output system was incorporated into the single-output Cd biosensor construct to provide additional information on biosensor functionality (i.e. growth and efficiency of gene expression). Incorporation of an additional fluorescence output is advantageous as it is less affected by environmental interference (i.e. dust and cell debris) and may provide a more sensitive measure of cellular metabolic activity. No significant decrease in mean RFP output for all multiplex Cd biosensors was observed until exposure to 400 μg ml^{-1} Cd(II), excluding LCR17(pBBc*adRgfp-rfp*) and MR-1(pBBc*adRgfp-rfp*) where a decrease was observed at 100 μg ml^{-1} Cd(II). Growth ($OD_{600\ nm}$) of DH5α(pBBc*adRgfp-rfp*), PAO1(pBBc*adRgfp-rfp*) and NCR3(pBBc*adRgfp-rfp*) was unaffected until exposure to 100 μg ml^{-1} Cd(II). In contrast the $OD_{600\ nm}$ of LCR17(pBBc*adRgfp-rfp*) and MR-1(pBBc*adRgfp-rfp*) decreased following exposure to 10 μg ml^{-1} Cd(II). A trend is apparent for all multiplex Cd biosensors with fluctuations in mean RFP output correlating with growth ($OD_{600\ nm}$) (Fig. 2b–f). The strict correlation between growth and RFP expression demonstrates that constitutive expression of fluorescent proteins can serve as an alternative means of monitoring cell viability. However, it is noteworthy that RFP expression and $OD_{600\ nm}$ varied between the various Gram negative bacteria tested. MR-1(pBBc*adRgfp-rfp*) had a significantly lower RFP output (< 6 RFU) in comparison to the other Cd(II) microbial biosensors (45–300 RFU), supporting the qualitative analyses. This further indicates that the multiplex Cd biosensor construct is transcribed differently between bacterial species. Interestingly, it was noted that while RFP decreased in correlation with $OD_{600\ nm}$, this was not observed with GFP expression, where a high IC was still observed at 400 μg ml^{-1} for the majority of the multiplex Cd biosensors. This may be accounted for by promoter strength where expression of the constitutively expressed *mrfp1* is directly related to the proportion of bacterial cells. In contrast, the Cd(II) responsive construct is highly expressed due to the higher influx of Cd(II) into the cell, although growth is affected. Benefit may be gained by further investigation of biosensors using methods, such as flow cytometry, to gain more insight into gene expression in individual cells. While monitoring fluorescence is not applicable *in situ* it may provide an initial starting point to develop alternative outputs, such as current production, which may be incorporated into future biosensor constructs to monitor biosensor functionality (Bereza-Malcolm and Franks, 2015; Biran et al., 2000; TerAvest et al., 2014).

3.3. The sensitivity, specificity and induction time of the multiplex cadmium biosensors varied upon exposure to heavy metals

The functional parameters of the five multiplex Cd biosensors were assessed to evaluate their potential to serve as a complementary analytical method for Cd(II) detection. A significant increase ($P < 0.05$) in GFP per $OD_{600\ nm}$, in comparison to the uninduced multiplex Cd biosensors, was used to determine the sensitivity, specificity and induction times. The IC was also determined (GFP per $OD_{600\ nm}$ of the induced sample, divided by the uninduced sample). Induction of bacterial cultures was between $OD_{600\ nm}$ 0.2–0.6 as no correlation was observed between the initial starting $OD_{600\ nm}$ of the culture and the detection limits of the multiple Cd biosensors. The limit of detection of the multiplex Cd biosensors are listed in Table 1. The PAO1(pBBc*adRgfp-rfp*) and NCR3(pBBc*adRgfp-rfp*) biosensors detected the lowest Cd(II) concentrations at 0.1 and 0.25 μg ml^{-1}, respectively. DH5α(pBBc*adRgfp-rfp*) also responded to low Cd (II) concentrations (0.5 μg ml^{-1}), however it was noted to have the most variable detection limit (higher standard error values), with the lowest statistically significant value being observed for 2 μg ml^{-1} Cd(II). MR-1(pBBc*adRgfp-rfp*) was the least sensitive, responding only to concentrations above 10 μg ml^{-1} Cd(II).

Variation in the detection limit of Cd biosensors has previously been observed (Ivask et al., 2009; Kumar et al., 2017; Tao et al., 2013; Tauriainen et al., 1998). For example, an *E. coli* MC1061 Cd biosensor harboring a *zntAlucFF* construct responded to 0.006 μg ml^{-1} Cd(II) (Ivask et al., 2002) while the Gram-positive *Staphylococcus aureus* RN4220 and *Bacillus subtilis BR151,* comprising *cadAlucFF*, were able to detect 0.001 μg ml^{-1} and 0.0006 μg ml^{-1} Cd(II), respectively (Tauriainen et al., 1998). A *cadCgfp* construct expressed in *E. coli* DH5α detected ~0.01 μg ml^{-1} Cd(II) (Kumar et al., 2017) while *cadRgfp* expressed in *E. coli* TOP10 detected 0.3 μg ml^{-1} Cd(II) (Tao et al., 2013). The low detection limits (0.0006–0.01 μg ml^{-1}) previously observed (Tao et al., 2013; Tauriainen et al., 1998) are indicative that the reporter and transcriptional regulator genes used affect the detection limit of microbial biosensors.

It is not readily reported that differences are also apparent between bacteria expressing the same construct. The different detection limits (Table 1) and varying IC between the multiplex Cd biosensors (Fig. 2a) in our study indicates that the bacterial species used also affects biosensor functionality. A 100-fold difference between detection limits was observed (0.1 and 10 μg ml^{-1}, respectively) of PAO1(pBBc*adRgfp-rfp*) and MR-1(pBBc*adRgfp-rfp*). As such, further investigation into the genetic basis for different detection limits between bacterial species may be of importance.

Specificity testing found the multiplex Cd biosensors were responsive to Hg(II) and Pb(II) [(excluding MR-1(pBBc*adRgfp-rfp*) which

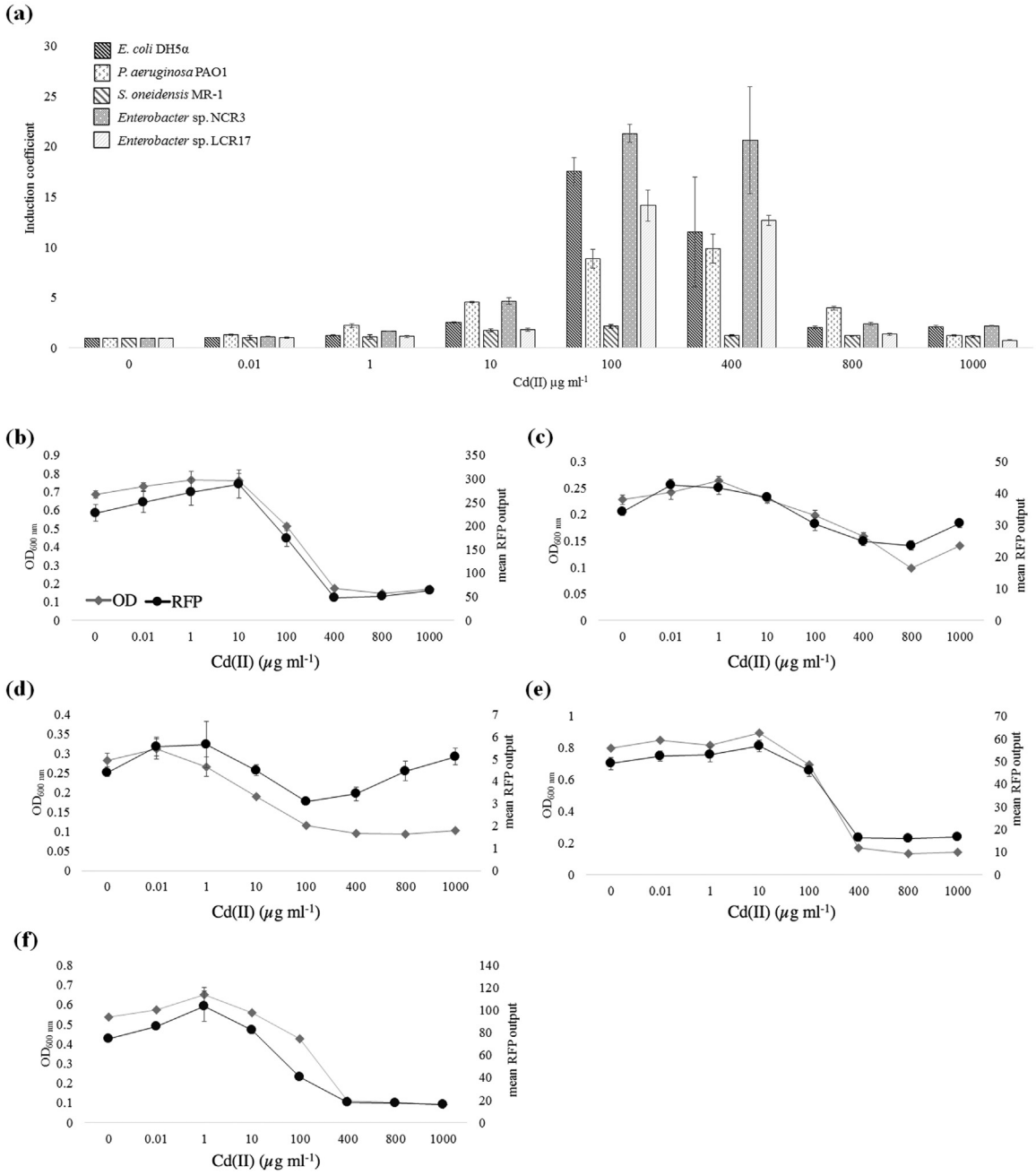

Fig. 2. The five multiplex Cd biosensors were exposed to a range of Cd(II) concentrations (0–1000 µg ml^{-1}) for 4 h. **(a)** An increase in GFP expression was observed from 1 to 100 µg ml^{-1} before a decrease upon exposure to higher Cd(II) concentrations (400–1000 µg ml^{-1}). A decrease in growth ($OD_{600\ nm}$) correlated with the higher concentrations of Cd(II) and is shown to decrease in a similar trend to RFP expression. **(b)** DH5α(pBB*cadRgfp-rfp*) **(c)** PAO1(pBB*cadRgfp-rfp*) **(d)** MR-1(pBB*cadRgfp-rfp*) **(e)** NCR3(pBB*cadRgfp-rfp*) **(f)** LCR17(pBB*cadRgfp-rfp*).

responded only to Hg(II)]. The IC for Hg(II) was higher in comparison to Cd(II), and in PAO1(pBB*cadRgfp-rfp*) the IC was 1.6 ± 0.1 and 2.6 ± 0.2, respectively. As CadR is similar to proteins from the MerR response regulator family which are responsible for mercury detoxification in bacterial systems, the response of the multiplex Cd biosensors to Hg(II) is unsurprising (Lee et al., 2001) as Hg(II) may bind more effectively to the CadR transcriptional regulator. DH5α(pBB*cadRgfp-rfp*) occasionally responded to As(III) which has not been previously reported (Table 1). Previously analysed Cd biosensors responded to other heavy metals including Hg(II) and Zn(II) (Ivask et al., 2002; Tauriainen et al., 1998) and functional analyses of the *cadAR* locus reported a response to Pb(II) (Lee et al., 2001). The multiplex Cd biosensors did not detect Zn(II), a transition metal similar in structure to Cd(II) and Hg(II) (Adriano, 2001; Krone et al., 2001). Interestingly, Zn(II) has previously elicited a response from the Cd biosensor genetic element used in this study (Lee et al., 2001; Tao et al., 2013). The differences in detection may be explained by the use of different zinc compounds between studies where the aforementioned studies used $ZnSO_4$, in comparison to $ZnCl_2$ used in this study.

It is possibly advantageous for transcriptional regulators to respond to several heavy metal ions as this would result in a selective advantage for the bacterial species. The noted differences between the multiplex Cd biosensors indicates that other factors also contribute to the specificity. Different uptake and efflux pathways between bacterial species along with binding affinity of different heavy metal compounds to the multiplex Cd construct would be expected to play a role. Differences in the kinetics of induction may provide further insight

Table 1.
The multiplex Cd biosensors were assessed to determine the limit of detection, response to other heavy metal ions and induction time.

Multiplex cadmium biosensor	Limit of detection		Response to other heavy metals (1 μg ml^{-1})		Time until induction [3 μg ml^{-1} Cd (II)]	
	Induction coefficient (IC)	Cd (II) (μg ml^{-1})	Induction coefficient (IC)	Heavy metal detected	Induction coefficient (IC)	Min.
E. coli DH5α(pBB*cadRgfp-rfp*)	1.3 ± 0.04 (2 μg ml^{-1})	0.5–2	1.53 ± 0.2; 2.5 ± 0.4; 3.21 ± 0.4; 1.6 ± 0.2	As(III), Cd(II), Hg (II), Pb(II)	7.64 ± 1.54	40
P. aeruginosa PAO1(pBB*cadRgfp-rfp*)	1.35 ± 0.06	0.1	1.6 ± 0.1; 2.6 ± 0.2; 1.6 ± 0.13	Cd(II), Hg(II), Pb (II)	2.7 ± 0.52	40
S. oneidensis MR-1(pBB*cadRgfp-rfp*)	1.54 ± 0.21	10	3.23 ± 0.8	Hg(II)	N.D.	N.D.
Enterobacter sp. NCR3(pBB*cadRgfp-rfp*)	1.62 ± 0.2	0.25	2.5 ± 0.4; 2.8 ± 0.02; 1.6 ± 0.04	Cd(II), Hg(II), Pb (II)	3.2 ± 0.4	20
Enterobacter sp. LCR17(pBB*cadRgfp-rfp*)	1.11 ± 0.05	1	1.61 ± 0.2; 3.1 ± 0.8; 1.4 ± 0.09	Cd(II), Hg(II), Pb (II)	2.44 ± 0.4	20

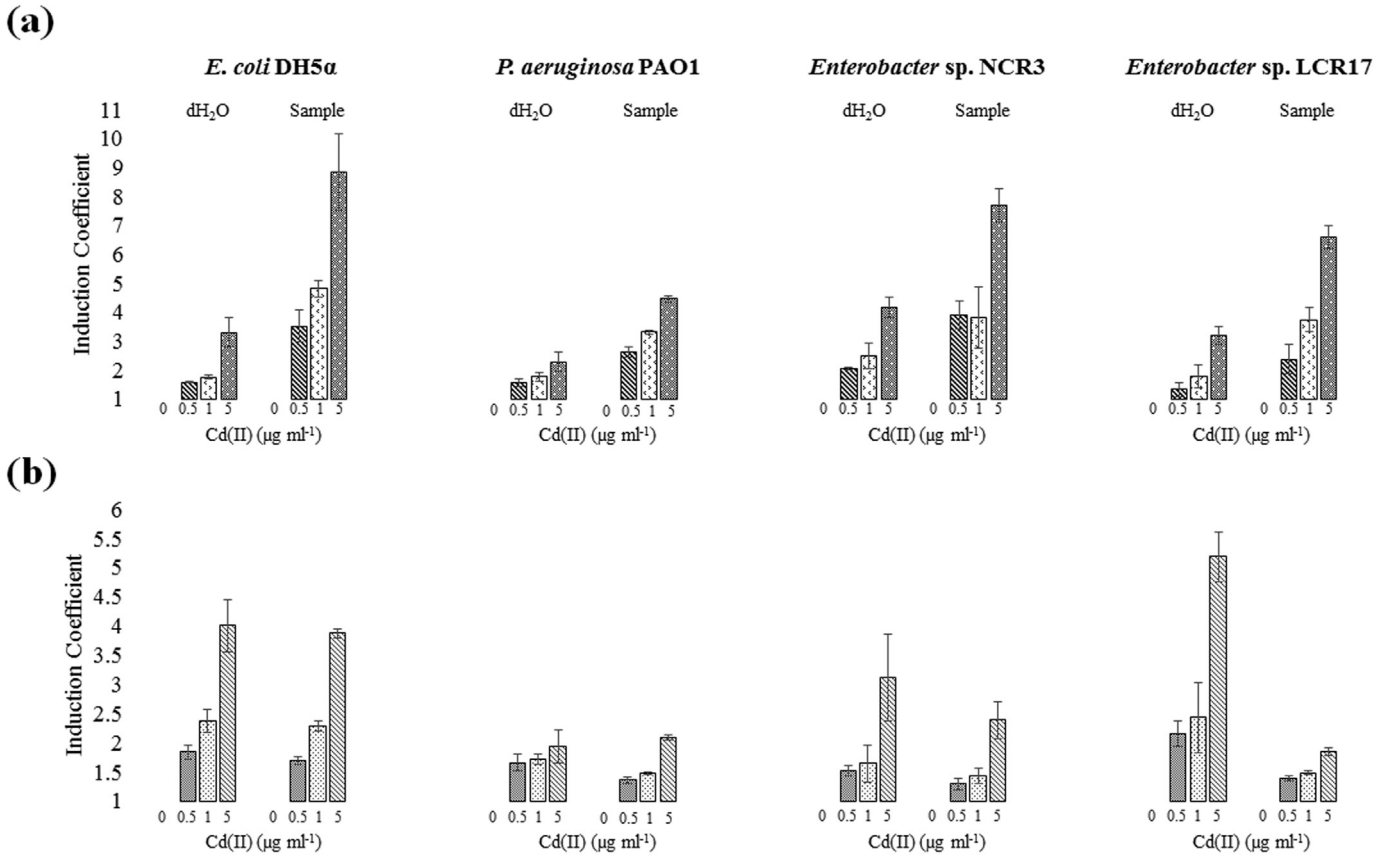

Fig. 3. Four multiplex Cd biosensors were exposed to either groundwater **(a)** or ocean water **(b)** samples with increasing Cd(II) concentrations (0–5 μg ml^{-1}) for 4 h. An increase in IC was observed for all multiplex Cd biosensors exposed to groundwater. In contrast the multiplex Cd biosensors were relatively unaffected by ocean water excluding LCR17(pBB*cadRgfp-rfp*).

into heavy metal binding efficiency and targets for genetic modification of transcriptional regulators for use in biosensors (Galvão and de Lorenzo, 2006; Park et al., 2005).

It is imperative that the multiplex Cd biosensors respond rapidly (i.e. induction time) to the presence of Cd(II) to provide real-time assessment. Thus to determine induction time, the GFP per $OD_{600\ nm}$ of the multiplex Cd biosensors was measured at 20 min intervals, following exposure to 3 μg ml^{-1} Cd(II). The concentration used was based on the lowest limit of detection (0.1–2 μg ml^{-1}) previously ascertained (Table 1). Due to low sensitivity and high variability observed in MR-1(pBB*cadRgfp-rfp*) during the initial analyses, it was omitted from subsequent analyses. The *Enterobacter* spp.(pBB*cadRgfp-rfp*) biosensors responded most rapidly, detecting 3 μg ml^{-1} Cd(II) within 20 min. DH5α(pBB*cadRgfp-rfp*) and PAO1(pBB*cadRgfp-rfp*) responded within 40–3 μg ml^{-1} Cd(II) (Table 1). The differences observed in the induction of the multiplex Cd biosensor may be due to the presence of heavy metal resistance mechanisms, including uptake systems. For example, a number of identified heavy metal resistance genes, including the *arsRDABC* operon, have been identified in NCR3 (Egidi et al., 2016). However, more likely is a difference in growth rate between bacterial species whereby slower growth may result in a decreased response time. Previously, the medium used has been reported to effect response time (Biran et al., 2000; Tauriainen et al., 1998), as such use of alternative media with different growth factors may increase growth rate and subsequently the response time of the multiplex Cd biosensors.

3.4. The multiplex cadmium biosensors were able to detect Cd(II) in environmental samples

To test the potential for *in situ* application the multiplex Cd biosensors [excluding MR-1(pBB*cadRgfp-rfp*)] were exposed to two environmental samples with varying Cd(II) concentrations. Groundwater and ocean water were collected from a Victorian lake system and Bass strait in Australia, respectively. These samples were chosen due to the widespread prevalence of Cd in natural water systems (WHO, 2011) and seafood (Bosch et al., 2016; Satarug et al., 2003). As such, it is necessary to test the ability of the multiplex Cd biosensors to detect Cd(II) in untreated (i.e. not autoclaved or filtered) water samples.

The multiplex Cd biosensors responded to varying Cd(II) concentrations (0–5 μg ml^{-1}) in the groundwater and ocean water samples (Fig. 3). Interestingly, the multiplex Cd biosensors exposed to groundwater (in comparison to those exposed to dH_2O) had a significantly higher IC (Fig. 3a), while mean RFP output remained unchanged (data not shown). The significantly higher IC of the multiplex Cd biosensors exposed to the groundwater sample may be explained by the presence of unknown inducers. As non-specificity of the multiplex Cd biosensors was previously observed (Table 1), it is not implausible that alternative inducers, such as heavy metals, may be the reason for the higher IC. In contrast, the IC of multiplex Cd biosensors exposed to ocean water and dH_2O were comparable [excluding LCR17(pBB*cadRgfp-rfp*)] (Fig. 3b). The IC for LCR17(pBB*cadRgfp-rfp*) exposed to ocean water and dH_2O containing 5 μg ml^{-1} Cd(II) was 1.85 ± 0.06 and 5.2 ± 0.4, respectively. As growth and mean RFP output were unaffected it is unclear why the IC differed. Although there was an increase in mean RFP output from the multiplex Cd biosensors exposed to ocean water, in comparison to dH_2O, it was not significantly higher except for DH5α(pBB*cadRgfp-rfp*) (data not shown). This may be due to fluctuations in gene expression as a consequence of stress response. Previously, an *E. coli* Cd biosensor was reported to be unaffected by the salinity of ocean water (Biran et al., 2000). Exposure of the multiplex Cd biosensors to groundwater or ocean water did not appear to affect growth ($OD_{600\ nm}$). However, in this study the observed effects of the untreated groundwater and ocean water samples differed between bacterial species. The gained insight into the functionality of the multiplex Cd biosensors indicates that developed microbial biosensors should be exposed to a range of environmental samples during analyses.

4. Conclusion

In this study, a novel multiplex Cd biosensor construct was developed and transferred into several Gram-negative bacterial species. The incorporation of the duplex fluorescent output allowed measurement of biosensor viability and functionality through use of fluorescence microscopy and slope agar plates. Functional parameters, including sensitivity, specificity and induction time, of the multiplex Cd biosensors were determined. *P. aeruginosa* PAO1(pBB*cadRgfp-rfp*) and *Enterobacter* sp. NCR3(pBB*cadRgfp-rfp*) had the lowest limit of detection of the five biosensors tested. The multiplex Cd biosensors were found to respond to multiple heavy metals, when exposed to 1 μg ml^{-1}, including Hg(II), Pb(II) and, in one case, As(III). All Cd biosensors responded rapidly to 3 μg ml^{-1} within an hour [excluding *S. oneidensis* MR-1(pBB*cadRgfp-rfp*)]. In addition, exposure of the multiplex Cd biosensors to environmental samples revealed further variabilities which may be attributed to unknown inducers in the samples. Overall, the differences noted in detection limits may not be just attributed to the genetic construct but can be linked to the differences between bacterial species including growth rate and native heavy metal resistance mechanisms. This study highlights the importance of testing biosensor constructs in several bacterial species and the usefulness of incorporating multiple outputs. As such, it is worthwhile for future biosensor constructs to be analysed in multiple bacterial species under a range of conditions. Subsequent genetic manipulation may then lead to development of improved microbial biosensors.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2017.03.029.

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