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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.