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Optimization of a Whole-Cell Cadmium Sensor with a Toggle Gene Circuit

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This work demonstrates improvement of a whole-cell cadmium detection sensor through construction of a gene circuit. A cadmium (II) specific regulatory promoter, P_{cadR}, from Psuedomonas putida 06909, is used in the assembly of a toggle circuit. The circuit contains the cadR promoter fused to lacI^q and gfp, and a divergently transcribed tac promoter and cadR. The toggle sensor exhibits lower background fluorescence, and a 20-fold lower detection limit in comparison to a nontoggle gene circuit. The detection limit of the toggle sensor is 0.01 μM (1.12 ppb) cadmium chloride, and tunable with the addition of isopropyl-b-D-thiogalactopyranoside (IPTG). The toggle sensor is highly specific to cadmium (II), and no response is elicited from zinc, lead, manganese, nickel, copper, and mercury. © 2009 American Institute of Chemical Engineers *Biotechnol. Prog.,* 25: 898–903, 2009
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Introduction

Heavy metal contamination has been a continual problem affecting human beings and wildlife. Predominantly from mine-drainage, soluble cadmium is dispersed into the environment where it is taken up by plants and bioaccumulated in higher food chain organisms. Major cadmium poisoning incidences include Japan's "Itai-Itai" disease,[1] and in the mine-belt region of the US where high concentrations of cadmium were found in the kidneys of willow ptarmigans.[2] Conventional detection methods such as atomic absorption spectrometry or inductively coupled plasma-atomic emission/mass spectroscopy are expensive, time intensive, and involve extensive sample preparation by trained personnel. The use of bacterial whole-cell sensors for detection of both organic and inorganic pollutants is a promising alternative to the conventional chemical methods. The advantages of using a bacterial sensing system include (1) ease of production, (2) simple measurement techniques, and (3) cost-effectiveness.

Whole-cell biosensors utilize naturally occurring bacterial promoters that respond to specific targets. Metal-responsive genes fused to reporter systems have been demonstrated to be effective biosensors. Several biosensors currently exist utilizing the natural bacterial metal resistant genes for detection of metals, such as arsenic,[3] mercury,[4,5] and cadmium.[6,7] As with all new methodologies, whole-cell sensing of metals poses several challenges, such as high background which decreases sensitivity,[8,9] nonspecificity,[6] and lack of environmental robustness.[10] In addition, the detection limit of the biosensor is often restricted by the natural affinity of the regulatory protein towards the metals.

Activation of the promoter by inducers occurs at the molecular level, thus providing potential for genetic manipulation to improve sensitivity and specificity. The field of whole-cell heavy metals sensors has made substantial advances in recent years as a result of bacterial genomic database expansion.[10,11] However, successful application of whole-cell sensors requires overcoming the aforementioned challenges. To alleviate the problem of low detection sensitivity, the approach of removing background signal has been applied.[8,9] A single copy of the repressor gene is often included in the expression vector, providing sufficient concentration of repressor to prevent expression before induction. Unfortunately, due to high level of the repressor concentration, induced promoter expression levels are typically dampened when compared with the clone expressing the vector without the repressor gene.[12]

The design and construction of synthetic gene circuits is the amalgamation of molecular biology with electrical circuitry. These circuits have enhanced our understanding of gene functions.[13–18] Cellular parts from different organisms are arranged in regulatory networks to perform complex logic functions. The responses of the biological circuit can be modeled with algorithms. An interesting and well-characterized gene circuit is the toggle switch, containing two divergently transcribed promoters each regulating a gene product that represses the other promoter.[19–21] When triggered by a specific stimulus, the switch exhibits a sigmoidal curve with distinctive low and high stable states. The rapid transition between the two states provides easily

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

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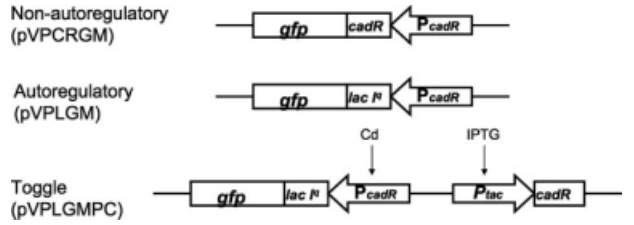

Figure 1. Diagram of the three genetic circuits: Non-autoregulatory (pVPCRGM), autoregulatory (pVPLGM), and Toggle (pVPLGMPC).

Cadmium induces the *cadR* promoter, and IPTG induces the *tac* promoter. For a detailed description of the circuitry, please refer to the supplementary material.

distinguishable "on" and "off" phases. This characteristic provides the possibility to construct a rapid, sensitive, and precise whole-cell biosensor for indicating presence or absence of specific analytes.

In this article, we demonstrate the use of a toggle genetic switch for optimization of a whole-cell sensor. A cadmium-specific *cadR* gene from the rhizobacterium *Psuedomonas putida* 06909[22] was used for the construction of the biosensor. Bacteria have acquired natural metal resistance operons such as the cadmium resistance system CadA. Cadmium resistance mechanisms include expression of p-type ATPase, cation-proton antiporter, and cadmium binding domains in the membrane or cytosolic domain. Most of the characterized cadmium resistance genes are from Gram-positive microbes, such as the *cadAC* gens in *Staphylococcus aureus* and *Listeria monocytogenes*. However, homologs of *cadA* are identified in both Gram-positive and Gram-negative organisms. Gram-negative homologs of *cadA* include *zntA*, found in *E. coli*. *ZntA* was originally thought to be a zinc-transporting ATPase; however, it is now known that it also confers resistance to lead and cadmium.[22] The *P. putida* cadmium resistance is regulated by *cadR* located adjacent to, but divergently transcribed from *cadA*. Psuedomonad cadmium resistance mechanism is generally uncharacterized. A recent research comparing DNA regulatory signals and protein sequences of eubacterial metal resistance regulons (e.g., *mer*, *cad*, *znt*, *hmr*, *pbr*, and *cue*) adds new insights to uncharacterized metal-specific loci and new metal-resistance genes.[23] Many bacterial cadmium resistance systems respond to multiple metals such as zinc and lead. However, Lee et al (2001) demonstrates that the *cadR* promoter only responds to cadmium.[22] The sequence homology of the *cadA* and *cadR* genes to the MerR family genes suggests that the mechanism for cations binding may be similar.[22,23] The trigonal coordinate geometry of the Hg^{2+} at the MerR binding site contributes to the specificity of the protein towards mercury.[24,25] It is thought that the CadR protein also has a tri-coordinate ligation with Cd^{2+},[23] which contributes to its high specificity.

The aim of this research is to demonstrate the use of gene circuitry for improvement of a cadmium (II)-specific biosensor using the *cadR* promoter and green fluorescent protein (GFP). The original host, *P. putida 06909*, is used to harbor the gene circuit containing the *cadR* promoter (P_{cadR}) regulating the $lacI^q$ and *gfp* genes, and the divergently transcribed *tac* promoter (P_{tac}) regulating the *cadR* gene (Figure 1). Thus, when cadmium is present, P_{cadR} is induced to produce LacI and GFP. LacI represses the P_{tac}, and GFP is used to monitor the level of P_{cadR} induction. When IPTG is added, P_{tac} induces production of CadR which in turn represses the P_{cadR}, and dampens GFP production. Inductions of the gene circuit by different concentrations of cadmium and six other cations have been conducted. The toggle biosensor exhibits lower background, higher signal-to-noise ratio, and improved sensitivity when compared with two other non-toggle gene circuits when cadmium is added. Furthermore, this improved biosensor is highly specific to cadmium (II) and does not respond to other valence II cations.

Materials and Methods

Bacterial strains, growth conditions, and plasmids

E. coli strains DH5a and XL1-Blue were used for construction and replication of the plasmids. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C. The rhizobacterium *P. putida* 06909 wild-type strain was maintained with 200 μg /mL ampicillin in LB. All *P. putida* sensor strains were grown at 30°C. The optical density of the cultures was measured at 600 nm (OD_{600}) using a spectrophotometer (DU640, Beckman). All molecular cloning techniques were performed according to Molecular Cloning Manual.[26]

Plasmid construction and transformation

Three gene circuits, pVPCRGM (non-autoregulatory), pVPLGM (autoregulatory), and pVPLGMPC (toggle) were constructed and characterized. For detailed description of plasmid construction, please refer to the Supplementary Material. The plasmids were first constructed in *E. coli*, then transformed into *P. putida*. The non-autoregulatory strain contained P_{cadR}, *cadR*, and *gfp*. The autoregulatory strain harbored a plasmid consisting of P_{cadR}, $lacI^q$, and *gfp*. The toggle strain harbored a similar plasmid consisting of P_{cadR} regulating $lacI^q$ and *gfp*, and a divergently transcribed P_{tac} regulating *cadR* (Figure 1). All *E. coli* transformants were selected on LB plates containing 50 μg/mL kanamycin, unless otherwise stated. Calf intestinal alkaline phosphatase (New England BioLabs) was added to all vectors in order to prevent self-ligation. Correct plasmids were confirmed by DNA sequencing at the Core Instrument Facility at the University of California, Riverside.

All transformation of *P. putida* 06909 was performed by electroporation using GenePulser Xcell (Bio-Rad Laboratories). Plasmid were electroporated into *P. putida* at 2.5 kV, 25 mF, and 200 Ω in 1-mm gap cuvets, grown in 1 mL SOC medium[26] without antibiotics for 2 h, and plated on LB plates with 100 μg/mL ampicillin and 50 μg/mL kanamycin.

Dose-response curves

A single colony from each of the *P. putida* strains was grown in 5-mL LB medium overnight. Cells were washed once with M9 medium [42 mM Na_2HPO_2, 24 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl, 1 mM $MgSO_4$, 0.4% glucose, 0.1 mM $CaCl_2$] and inoculated into M9 medium containing 10 μg/mL kanamycin to OD of 0.1, and grown in a shaker incubator at 30°C until mid-exponential phase (OD $\sim$ 0.4). Cadmium stock solutions were added to the culture at 1,000 times dilution to obtain the final concentration range of 0.001 to 1 μM. After 4 h growth, the fluorescence intensity of the cell cultures was measured using a fluorometer (POLARStar Optima, BMG), with an excitation wavelength at 480 nm and emission wavelength of 520 nm. Optical

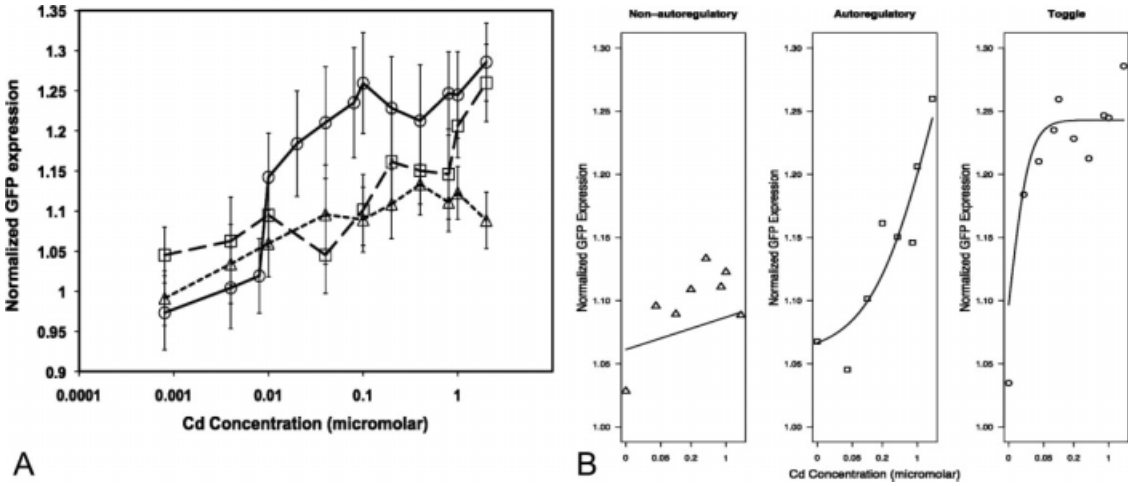

Figure 2. Dose response curves of all three circuits (A) Normalized GFP expression vs. cadmium concentrations and (B) Fitted dose response curve to each of the gene circuits: pVPCRGM-non-autoregulatory of the three gene circuits: pVPCRGM-non-autoregulatory (Δ), pVPLGM-autoregulatory (□), and pVPLGMPC – toggle (○).

Each data point is the mean of six replicates, and the error bar represents the standard error of mean (S.E.M.).

density was measured with a spectrophotometer at 600 nm. To obtain normalized GFP expression, all fluorescence intensity values were divided by the optical density of the cells and scaled by division of the background fluorescence obtained without inducers (metals and IPTG). Normalized GFP expression values for all figures were calculated in the same fashion.

Inhibition of biosensor with IPTG

To demonstrate the functionality of the *tac* promoter, dose-response experiment with IPTG was performed. Protocol for cell growth was the same as previously described, except that 0.4 μM cadmium chloride was added simultaneously with concentrations of IPTG ranging from 0.1 to 2.4 mM. Results were normalized as described in the previous section.

Specificity of toggle sensor

The specificity of the toggle sensor for cadmium was investigated with six metal cations. The growth condition was the same as for the generation of the dose response curves. The cations were added at exponential growth phase and grown in a shaker incubator for 3 h. The concentrations and cations used for induction were: 50 μM $ZnCl_2$, 10 μM $Pb(C_2H_3O_2)_2$, 25 μM $MnCl_2$, 25 μM $NiSO_4$, 25 μM $CuSO_4$, 0.5 μM $HgCl_2$, and 0.4 μM $CdCl_2$. Results were normalized as described earlier.

Statistical analysis

The *drc* package[27,28] in the statistical software **R** (R Development Core Team 2008[29]) was used to fit dose response curves for the biosensors. The autoregulatory and toggle curves were fitted to the four-parameter Weilbull model using the *drc* package. The non-autoregulatory curve was fitted to the general linear model equation in the *stats* package. Microsoft Excel was used for data management and calculations of means and standard errors.

Results

Toggle circuit design

The toggle circuit consisted of the *cadR* promoter fused to the *lacI*^{q} and *gfp* genes, with the *tac* promoter and *cadR* gene transcribed divergently on the same broad-host-range plasmid (Figure 1). The repressors, *lacI*^{q} and *cadR*, inhibited the *tac* and *cadR* promoters, respectively. For comparison, the non-autoregulatory sensor contained the *cadR* promoter, *cadR* and *gfp*, and the autoregulatory sensor contained only the *cadR* promoter, *lacI*^{q} and *gfp* genes. *P. putida* 06909 was used as host for the expression of these plasmids.

Cadmium-induced GFP expression and fitted dose-response curves

To investigate the detection limit and the dynamic range of the three strains, GFP fluorescence intensity vs. cadmium concentration was obtained (Figure 2A). $LacI^q$ was included in the autoregulatory strain to allow for direct comparison to the toggle strain. The detection limit is defined as the lowest cadmium concentration where the GFP expression is greater than the upper standard error limit of the background sample GFP expressions. For the non-autoregulatory and autoregulatory biosensors, a gradual increase in dose responses was observed. The toggle biosensor exhibited a sharp increase in GFP expression. The non-autoregulatory strain had a detection limit of 0.08 μM cadmium. The detection limit for the autoregulatory strain was found to be at 0.2 μM. The detection limit of the toggle strain was observed to be at 0.01 μM. The detection limit of the non-autoregulatory and autoregulatory strains are 8-fold and 20-fold higher than the limit obtained with the toggle biosensor, respectively. This higher detection limit of the non-toggle strains could be an attribute of the auto-induced fluorescence at low cadmium concentrations. Whereas the GFP expression of the toggle strain was lower than the GFP expression of the non-toggle strains at 0.008 μM cadmium.

Models were fitted to the data sets in order to compare the dose response curves of the three systems (Figure 2B). The non-autoregulatory data did not fit well to the nonlinear

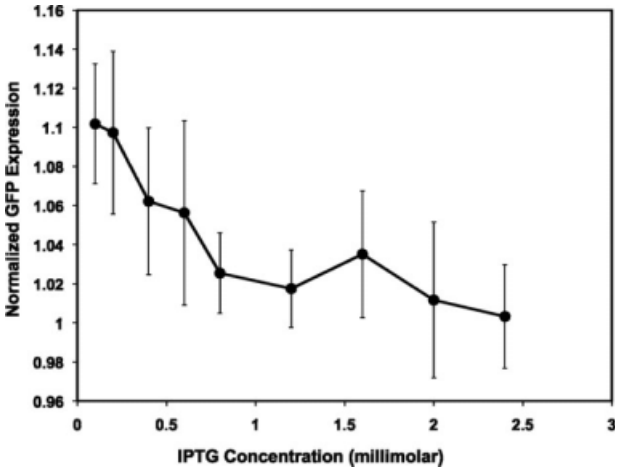

Figure 3. Inhibition with IPTG in the presence of 0.4 μM cadmium chloride.

Normalized GFP expression of the toggle sensor vs. IPTG concentration. Each data is the mean of six replicates, and the error bar represents the standard error of mean (S.E.M.).

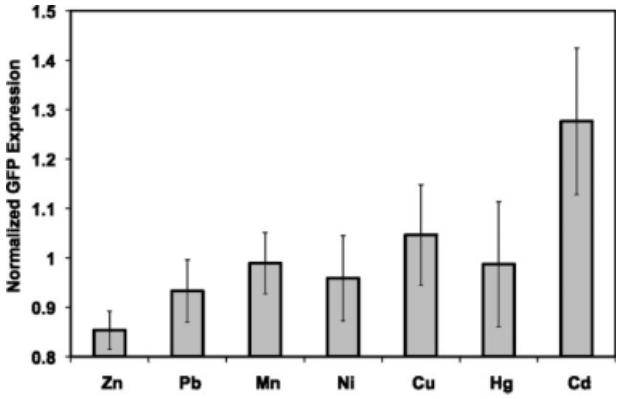

Figure 4. Specificity of the toggle sensor.

The mean of the cadmium induced cells is statistically different from mean GFP values from cells induced with other cations (P-value ≤ 0.04; one-tail t-test). The concentrations and cations used for induction were: 50 μM $ZnCl_2$, 10 μM $Pb(C_2H_3O_2)_2$, 25 μM $MnCl_2$, 25 μM $NiSO_4$, 25 μM $CuSO_4$, 0.5 μM $HgCl_2$, and 0.4 μM $CdCl_2$. Each data point is the mean of four independent replicates, and the error bar represents standard error of the mean (S.E.M.). The experiment was repeated twice with similar results.

model, and was fitted to the general linear model with a slope of 1.086 (P-value <0.001). The autoregulatory data set was fitted to the four-parameter Weibull model.[27] A slope of 0.68 with a P-value of 0.036 were obtained. The toggle data was also fitted to the four-parameter equation, and a slope of 2.24 with a P-value of 0.053 were obtained. The effect dose (ED10) where 10% of GFP response was calculated. The ED10 for the autoregulatory and toggle strains were 0.084 μM and 0.004 μM cadmium concentrations, respectively.

Inhibition of fluorescence with IPTG

To compare promoter strengths and to demonstrate tunability of the toggle circuit, GFP expression was investigated in the presence of 0.4 μM of cadmium and IPTG concentration ranging from 0.1 to 2.4 mM. Increasing IPTG concentration corresponded to decreased GFP expression even in the presence of cadmium (Figure 3). IPTG concentration of above 0.8 mM did not further inhibit GFP expression. Thus, at 0.8 mM IPTG, the toggle sensor was switched to the "off" state.

Specificity of cadR *promoter toggle sensor*

The specificity of the toggle biosensor was investigated in the presence of six different cations. Cultures with cadmium yielded GFP intensity significantly higher than cultures with the other cations (Figure 4). There were no statistically significant differences among GFP intensities due to the addition of lead, manganese, nickel, copper, and mercury. The minor increase in GFP expression with copper addition was not significant and accounted for by sample error.

Discussion

P. putida 06909 is an isolate from citrus root, and exhibits *cad* operon-encoded cadmium resistance. To take advantage of its high specificity towards cadmium (II),[22] the *cadR* promoter from *P. putida* was selected for construction of a whole-cell biosensor. The *cadR* gene is autoregulatory in nature where the control of the transcript is by its own product. Therefore, there is always a basal amount of CadR present in the cells. Initially, a cadmium (II)-specific whole-cell biosensor was constructed using *P. putida* 06909 harboring a plasmid containing the *cadR* promoter fused to the *gfp* gene. However, due to the presence of the *cad* operon on the chromosome, high background fluorescence was observed. The over-expression of the CadR repressor through a second plasmid containing P_{tac}-*cadR* was used in an attempt to lower background fluorescence. However, unlike results from Neufeld et al.[8] where over-expression of the repressor successfully reduced the background without affecting the sensitivity, expression of CadR completely inhibited the activity of the *cadR* promoter (data not shown). In addition, a mutant strains of *P. putida* 06909 with *cadR* gene knockout[22] was also used as host in hope of decreasing background. However, the strain without the *cadR* gene lost its cadmium resistance, and growth was inhibited with addition of 0.1 μM cadmium. Therefore, a strategy using synthetic gene circuits was formulated to mitigate the encountered challenges.

The toggle gene circuit improves the cadmium biosensor by decreasing fluorescence background, lowering the detection limit by 20-fold, and increasing the signal-to-noise ratio. One feature of the toggle biosensor is the steep increase in GFP intensity at 0.01 μM (1.12 ppb) cadmium concentration (Figure 2A&B). This bi-phasic characteristic of the toggle dose-response curve clearly denotes the concentration of cadmium where the biosensor was "on" or "off." In contrast, the non-autoregulatory and autoregulatory strains exhibit a more gradual incline with increasing cadmium concentration. The autoregulatory strain reaches similar GFP expression at 1 μM cadmium concentration to the toggle strain, whereas the GFP expression of the non-autoregulatory strain was much lower and began to decline at 1 μM.

The toggle strain dose response slope (2.24) is 3.3-fold higher, and thus steeper than the autoregulatory strain slope (0.68) (Figure 2B). This result indicates toggle gene circuit's faster response to increasing cadmium concentration. The ED10 of the toggle strain is 21-fold less than that of the autoregulatory strain. The difference between the dosages eliciting 10% of the observed GFP expression from the two

strains is consistent with the difference observed in detection limits.

No influence of the native *cad* operon on the reporter system is expected. A 10–20 copy-number plasmid, pVLT33, was used to express the gene circuits; therefore, the production of CadR by the plasmid is higher than the inherit CadR concentration from one chromosomal copy.

The concentration of IPTG used to switch "off" the biosensor corresponds to the established range (0.8–1 mM) used in regulating the *tac* promoter for bacterial gene expression.[26] This result validates the central feature of the toggle circuit, indicating that both *cadR* and *tac* promoters in the gene circuit contribute to GFP expression and could be used for fine-tuning of the gene circuit behavior. Therefore, replacing the *tac* promoter with another that has tighter control of the *cadR* repressor could yield more sensitive cadmium detection limit.

The minimal inhibitory concentration (MIC) of cadmium for *P. putida* 06909 is 1.7 mM when grown on mannitol-glutamate agar supplemented with yeast extract.[22] The concentrations of cadmium (0.001–1 μM) used in this study were three orders of magnitude below the MIC of *P. putida* 06909, and the cell densities for the dose-response experiments were monitored to ensure that the amount of cadmium added was at sub-inhibitory concentration. Therefore, cadmium toxicity to the cells did not influence whole-cell detection capability. The inherent cadmium resistance in *P. putida* is advantageous for its ability to survive under high cadmium concentration.

A previously reported *E. coli* sensing system, with CadA and CadC regulatory proteins fused to GFP, responded to cadmium, lead, and zinc ions.[6] The CadC-GFP sensor had a cadmium detection range from 10 nM–10 μM; however, the fluorescence intensity decreased sharply at higher cadmium concentration due to cadmium toxicity.[6] Unlike the *cadCA* sensor, the *cadR* toggle sensor was highly specific and solely induced by cadmium. The specificity of the *cadR* promoter indicates a fundamentally different cation recognition mechanism than the CadCA regulatory proteins. Even though the *E. coli* CadC-GFP biosensor has a greater dynamic range of GFP intensity, the lack of specificity and toxicity at cadmium concentration above 10 μM makes it non-ideal for detection of environmental samples. Environmental cadmium concentration at a mined ore contaminated site is reported to be above 15 mg per kg (133.9 μM) and the site also contains lead and zinc.[30] It is important to construct a biosensor capable of distinguishing cadmium from zinc or lead in order to truly access health risks. In addition, a sensor capable of striving under high metals concentrations and low nutrient conditions is necessary. This *P. putida* strain has a 1.7 mM minimal inhibitory concentrations (MIC) towards cadmium chloride, whereas *E.coli* has an MIC of 0.2 mM.[22] The *P. putida* cadmium biosensor has a 8.5-fold higher tolerance towards cadmium than *E. Coli* and will be better suited for environmental samples with elevated cadmium concentrations. An engineered strain of *P. putida* 06909 with the ability to accumulate cadmium was inoculated onto sunflower roots grown in hydroponic solution containing 80 μM cadmium chloride, and the population was stably maintained for at least 27 days without addition of bacterial growth medium.[31] Furthermore, the *P. putida* sensor has the potential to serve as a simple pass or fail indicator once the circuit is tuned to cadmium environmental standards. Under certain situations, the knowledge of whether cadmium exceeds a threshold is sufficient for a quick decision for further analysis or mitigation measure.

Conclusion

This report demonstrates the use of a toggle genetic circuit to optimize a sensitive, specific, and robust whole-cell biosensor for cadmium detection. The toggle circuit lowers the background fluorescence, and increases detection sensitivity for cadmium by 20-fold. The distinct difference between the "on" and the "off" states of the toggle sensor and specificity are ideal for indication of presence or absence of cadmium. This research paves the way for a novel methodology of optimizing biosensors. Even though, the capability of this cadmium sensor requires further improvement, the result demonstrates the usefulness of synthetic biology for whole-cell detection. Accumulation of low concentrations of cadmium leads to chronic toxicity such as cancer,[32] thus it is imperative to detect cadmium at the nanomolar or parts per billion concentration. The detection limit of this whole-cell sensor is 0.01 μM ($\sim$1.12 ppb), which is lower than the 5 ppb maximum contaminant level goal set by the US Safe Drinking Water Act of 1974 (http://www.epa.gov/OGWDW/contaminants/dw_contamfs/cadmium.html). This biosensor has promising potential for future development into a rapid and specific screening tool for detection of cadmium in water.

Acknowledgments

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