Gene circuit engineering to improve the performance of a whole-cell lead biosensor

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Abstract

To improve the performance of a whole-cell biosensor for lead detection, we designed six gene circuits by re-configuring the regulatory elements and incorporating positive feedback loops to the circuits. The lead resistance operon *pbr* encodes six genes with *pbrRT* on one side of the promoter and *pbrABCD* on the other side. PbrR, the divergent promoter it regulates, and GFP were used to design the lead biosensors. One has *pbrR* and *gfp* on opposite sides of the promoter mimicking the native operon. We re-configured it by placing *pbrR* and *gfp* on the same side or under two separate promoters. The one with *pbrR* and *gfp* on the same side demonstrated lead sensitivity 10 times higher

than the others. Positive feedback loop was introduced to these circuits. The strength of the output signal from the designs with positive feedback loop was 1.5-2 times stronger than those without positive feedback. This study demonstrates the importance of configuration and positive feedback as effective strategies to improve the performance of lead biosensors and they can be extended to the design of other whole-cell biosensors.

Keywords

Configuration; Heavy metal detection; Positive feedback amplifier; Promoter configuration; Sensitivity; Whole-cell biosensor

Introduction

Heavy metal pollution and human exposure to toxic heavy metals have dramatically risen due to their increasing utilization in industry, agriculture, medicine, as well as domestic and technological applications. Due to their bioaccumulative properties, toxic heavy metals, such as lead, mercury, and cadmium, could cause severe health problems even at low exposure levels (Bruins *et al.*, 2000). Taking lead as an example, metallic lead and its compounds are stable and hard to remove via metabolism, thus through food chain accumulation lead causes serious damage to the nervous system, reproductive system, and blood system, especially for children (Hung *et al.*, 2010, Wang *et al.*, 2015). Owing to its subsistent damage to human, it has become crucial to develop accurate, sensitive, and selective assays for the detection of lead.

Until now, plenty of classical analytical methods used for lead detection, including atomic adsorption spectroscopy (AAS) (Chen & Teo, 2001) and inductively coupled plasma-mass spectroscopy (ICP-MS) (da Silva *et al.*, 2000, Oliveira *et al.*, 2011), show high sensitivity, but require expensive equipment and years of special training. Moreover, these methods are time-consuming and require the transport of samples to special laboratories. Some chemical methods, which are relatively easier to operate, lead to the production of other toxic species and secondary environmental pollution (Ruengsitagoon *et al.*, 2010). Therefore, the development of low-cost biosensors suitable for rapid *in situ* detection of heavy metals has attracted increasing attention in recent years.

Biosensors using small chemical molecule probes (Xiao *et al.*, 2007, Zuo *et al.*, 2009) and biological colorimetric probes (Liu & Lu, 2003) opened the door for the rapid and convenient detection of lead. Moreover, fluorescent lead probe based on unique lead regulatory protein toward lead is about 1000 times over other metal ions (Chen *et al.*, 2005). However, the application of these biosensors suffers from inadequate sensitivity and poor stability. Microbial whole-cell biosensor, based on the genetic elements in response to the chemical species to be detected, is a prospective method for the detection of heavy metal ions. In the past years, whole-cell biosensors have been designed to detect a wide range of heavy metals, such as zinc, copper, arsenic, lead and mercury (Magrisso *et al.*, 2008, Bereza-Malcolm *et al.*, 2015). The basic design typically utilizes genetic regulatory elements rendering cells resistant to heavy metals and couples the expression of a reporter gene, often fluorescence, luminescence, or enzyme assays, to the concentration of heavy metals.

For the specific detection of lead, the regulator PbrR and its cognate promoter from the lead resistance operon *pbr* in *Cupriavidus metallidurans* CH34 are typically used to express a reporter gene in response to lead. The *pbr* operon, located on the pMOL30 plasmid of *C. metallidurans* CH34, is

composed of *pbrR*, encoding a MerR-type regulatory protein PbrR, and structural genes *pbrA*, *pbrB*, *pbrC*, *pbrD*, *pbrT* (Mergeay *et al.*, 1985, Mergeay *et al.*, 2003). The gene *pbrT*, encoding a lead uptake protein, is located downstream of *pbrR*. The genes *pbrABCD*, located on the other side and transcribed in the opposite direction, encode the P-type lead efflux ATPase, the predicted integral membrane protein, the predicted prolipoprotein signal peptidase, and the lead sequestration protein, respectively (**Fig. 1a**) (Borremans *et al.*, 2001, Taghavi *et al.*, 2009). PbrR dimer binds to the divergent promoter at the center of the operon. In absence of lead, the expression of all genes is off. While lead is present, the binding of the lead ion to PbrR changes the conformation of the protein-DNA complex, resulting in the state of promoter being changed to open and subsequent expression of all six genes, *pbrRT* and *pbrABCD* (Brown *et al.*, 2003).

Effort has been made to construct genetic circuits for lead sensing by directly associating PbrR and its promoter with reporter genes, such as luminescent or fluorescent proteins. The performance of the whole-cell lead biosensors has been examined and improved by optimizing growth media, reporter genes, and host organisms. The first PbrR based whole-cell biosensor was built by Corbisier and colleagues in its native host *C. metallidurans* CH34 using luminescence as the reporter. They developed a minimal medium to reduce lead chelation and allow maximal lead bioavailability and thus a higher expression of luminescence at low lead concentrations (Corbisier *et al.*, 1999). Later fluorescent protein reporters such as GFP and RFP were used instead for more convenient detection and stronger output signals (Chakraborty *et al.*, 2008, Wei *et al.*, 2014, Bereza-Malcolm *et al.*, 2016). While *C. metallidurans* CH34 was initially used as the host (Corbisier *et al.*, 1999), it was shown the PbrR-*pbr* promoter functions well in other gram-negative bacteria. Chakraborty *et al.* designed a PbrR-promoter-GFP based biosensor in *E. coli* and Wei et al. also used *E. coli* as the host but used RFP

instead (Chakraborty *et al*., 2008, Wei *et al.*, 2014). Bereza-Malcolm *et al.* further compared five commonly used Gram-negative bacterial hosts and demonstrated that the *Pseudomonas* lead biosensor performed best and was able to detect lead below the WHO guideline level 0.01 μg mL^{-1} (Bereza-Malcolm *et al.*, 2016). Although the optimal growth conditions of non-native hosts differ from *C. metallidurans* CH34, the PbrR based biosensors function well in the non-native hosts.

All of these lead whole-cell biosensors are based on the genetic circuit composed of PbrR, P_{pbrRT}, and reporter. Genetic circuits have been designed and engineered to achieve various functions such as feedback circuits and feedforward loops (Brophy & Voigt, 2014). Regulatory feedbacks are often introduced to enhance the performance of artificial genetic circuits. Negative feedbacks have been shown to reduce the noise level while positive feedback loops have demonstrated memory, enhanced specificity and output signals, and increased noise level (Sayut *et al.*, 2006). A positive-feedback amplifier using a variant of the quorum-sensing regulator LuxR renders the genetic circuits more sensitive to the effectors and/or amplifies the output signal (Nistala *et al.*, 2010). Though it has been applied for detecting antibiotics and nutrients, no research has been done to study the effect of genetic circuit engineering on heavy metal biosensors. In this work, we will incorporate the positive feedback loop into the lead sensing circuits to improve its performance. This is the first time positive feedback is used in constructing genetic circuits for heavy metal detection.

Configuration of promoters is also an important design parameter. The *pbr* operon takes a divergent configuration, by which *pbrRT* and *pbrABCD* are transcribed in opposite directions from the divergent promoter, while in operons with similar mechanisms such as the arsenic resistance operon *ars*, all of the genes are expressed from the same side of the promoter (Wu & Rosen, 1993). Divergent promoter is common in native systems and often associated with transcription factors (Yamada *et al.*,

2003, Lepoivre *et al.*, 2013). Over 600 operons are arranged in a divergent configuration involving about 25% of all genes in *E. coli* (Salgado *et al.*, 2013, Masulis *et al.*, 2015). Studies of this intriguing genetic configuration suggest that it allows for the differential regulation of gene expression or couples gene expression from both sides (Wang *et al.*, 2011). It may also allow simultaneous transcription of genes from both sides and speed up gene expression compared with genes from a unidirectional promoter. Genetic circuits with the divergent configuration have been shown to have enhanced sensitivity to the inducer compared to circuits arranged in other configurations with the same regulatory mechanism using the *tetRA* operon as an example (Wu & Rao, 2010). Although the effect of configuration on a genetic circuit depends on the specific regulatory elements, the configuration of a genetic circuit should be taken into account as a design parameter in genetic circuit engineering. The role of the divergent configuration of the *pbr* operon is not clear, and thus in this work, we will design the genetic circuits in different configurations and explore the role of divergent configuration in the whole-cell lead biosensor.

In summary, the performance of the whole-cell lead biosensor could be improved by genetic circuit engineering. In this study, six lead biosensors with different configurations were designed and compared in their response to lead. Compared with previous research, this study has two novel features: (i) gene circuits with different configurations were compared to investigate the role of divergent configuration in the *pbr* operon; (ii) a positive-feedback amplifier was introduced to enhance the performance of the lead biosensor. Lead biosensors with different configurations demonstrated distinguishing response for the detection the lead, and the biosensors with positive-feedback amplifier gave stronger output signals. Hence, this work provides a novel strategy for the design of lead whole-cell biosensors, which will aid in developing new environmental

monitoring techniques and improving the performance of existing techniques for the heavy metal ions and other contaminants.

Materials and methods

Media, growth condition, and bacterial strains

Luria-Bertani (LB) broth (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride) was used for the growth of bacteria. All experiments were performed at 37°C. Kanamycin was used at 50 μg mL^{-1} and chloramphenicol was used at 25 μg mL^{-1} when needed.

E. coli DH5α was used as the host for the construction of plasmids and subsequent characterization of the designed biosensors.

Plasmid construction

All plasmids and strains used in this study were listed in **Table 1**. The primers used in this study were listed in **Table S1**. The plasmid pET28a were used for the construction of the biosensors.

The lead sensing plasmid pABG was constructed in two steps. Firstly, the DNA fragment (*ΔpbrA*-P_{pbrRT}-*pbrR-ter*) was amplified from the plasmid pUC57-G7-Kan with primers 1F and 2R, and then inserted into pET28a using the restriction sites BglII and BamHI, forming the plasmid pA. Secondly, the DNA fragment including *ΔpbrR* and the promoter $P_{pbrABCD}$ was amplified from pUC57-G7-Kan using 3F and 4R, and *gfp* was amplified from pUC57-gfp-Kan using primers 5F and 6R. The resulted two fragments were joined together using 3F and 6R via PCR SOEing and inserted into pA between BamHI and XhoI, giving the plasmid pABG.

The plasmid pABR was constructed using in two steps. First, the *luxR* gene was amplified with primers 7F and 8R using pGN12 as the template. Second, the DNA fragment (*ΔpbrR*-$P_{pbrABCD}$) and the *luxR* gene were joined together using primers 3F and 8R via PCR SOEing and inserted into the BamHI and XhoI on pA, yielding the plasmid pABR.

The DNA fragment (*ter-pbrR*-$P_{pbrABCD}$) was amplified from pUC57-G7-Kan using primers 9F and 10R and inserted into pET28a at the BglII and BamHI sites, giving the plasmid pC. Then *gfp* was amplified using primers 11F and 6R and *luxR* was amplified using 12F and 8R, and the two genes were inserted into pC between BamHI and XhoI, yielding pCG and pCR respectively.

The plasmids pDG and pDR were constructed in a similar way. The DNA fragment (*ΔpbrA*-P_{pbrRT}-*pbrR*) was amplified from pUC57-G7-Kan using primers 1F and 13R and then inserted into pET28a at the BglII and BamHI sites, giving the plasmid pD. Then *gfp* was amplified using 14F and 6R and *luxR* was amplified using 15F and 8R, and the two genes were inserted into pD between BamHI and XhoI, yielding pDG and pDR respectively.

Growth curves of all biosensors

The constructed plasmids were transformed into *E. coli* DH5α cells, forming six whole-cell biosensors.

To assess the growth of six biosensors as well as DH5α, strains were grown overnight and then subcultured in fresh LB media. When the optical density reached 0.6, $Pb(NO_3)_2$ were added to different final concentrations (0, 5, 50, 100, 200 and 300 μM) and optical density at 600 nm was measured every 2 hours.

Response assay of all biosensors with time

Biosensor cells at the OD_{600} of 0.6 were induced with 5 μM $Pb(NO_3)_2$. 1 mL culture was taken every two hours for the measurement of optical density and fluorescence. HITACHI F-2500 Fluorescence Spectrophotometer was used for the measurement of fluorescence (with the excitation at 488 nm and emission at 525 nm). Three independent replicates were conducted, and the average fluorescence per OD_{600} was calculated to obtain fluorescence intensity.

Dose-response assay of all biosensors

An overnight culture was diluted 1:100 in 3 mL fresh LB medium to start the subculture. When the optical density reached 0.6, the biosensors were exposed to $Pb(NO_3)_2$ at different concentrations (0, 0.001, 0.01, 0.1, 1, 5, 10, 20, 50, 100, 200 and 300 μM) for 8 hours. The optical density and fluorescence of triplicates were measured.

Results and discussion

Design and construction of lead biosensors

Based on the regulatory mechanism of the *pbr* operon, we rationally designed three self-feedback biosensors S21, S22, and S23, using the regulator PbrR, the divergent promoter, and the reporter gene *gfp*. Compared with the wild-type GFP protein from *Aequorea victorea*, a GFP mutant protein with three amino acid substitutions S2A, F64L, S65T was used in this study, which showed only one excitation maximum at 489 nm and emission spectrum near 510 nm (Fuhrmann *et al.*, 1999). As shown in **Fig. 1b**, the three self-feedback biosensors are the same in terms of the regulatory mechanism but arranged in different configurations. S21 has *pbrR* and *gfp* expressed from two

separate promoters. S22 is similar to the native operon, in which *pbrR* and *gfp* are expressed in a divergent way. In S23, *pbrR* and *gfp* are arranged on the P_{pbrRT} side of the divergent promoter in a tandem pattern. In order to improve the performance of the biosensors, a positive-feedback amplifier enabling the amplification of the signal response was introduced into the designed gene circuits to form new ones named D41, D42, and D43. In the construct of the amplifier, $LuxR\Delta_{2\text{-}162}$ acts as a constitutive activator, which activates transcription of the genes under the control of the promoter P_{luxI}. A second $LuxR\Delta_{2\text{-}162}$ is placed downstream of the *pbrR* promoter. After activating transcription, the $LuxR\Delta_{2\text{-}162}$ feeds back into its own promoter, leading to the signal amplification (Nistala *et al.*, 2010).

Growth curve of all biosensor strains

High concentrations of lead inhibits cell growth by affecting enzyme activity, damaging membrane function and altering the osmotic balance possibly, thus the upper detection limit of the biosensors is restricted by their tolerance to lead. The growth curves were measured at different concentrations of lead ions. The growth curves of DH5α were shown in **Fig. 2** and those for the lead biosensors were shown in **Fig. 3**. Growth of DH5α in LB media was slightly affected when the concentration of lead went up to 50 μM and significantly inhibited when the lead concentration was 100 μM or above. In comparison, the growth of the six lead biosensors was only affected when the lead concentration was above 100 μM. The generation time of each strain under different growth conditions were calculated and shown in **Table S2**. When lead is at a concentration of 100 μM or lower, the generation time of all six biosensors are similar to that of DH5α. At 300 μM, the generation times of the six biosensors are much longer, ranging from 48.2 to 60.0 min and that of DH5α is even longer, about 164 min, suggesting it is more severely impaired by lead at high concentrations. The presence of the lead

sensing genetic circuit enhanced the lead tolerance of the cells, probably attributed to the bound of lead to the PbrR and thus lower concentration of remaining lead.

In this work, the LB rich medium was used for cell growth in presence of lead. One concern using rich media is the possible chelation of lead and the decrease of available lead to the biosensor. Corbisier *et al.* developed a RM minimal medium to minimize lead chelation which enhanced the expression level of the reporter at low concentrations of lead (Corbisier *et al.*, 1999). On the other hand, the improved lead availability using this medium could cause a more significant inhibition of bacterial growth at high concentrations of lead. In order to detect lead at a wide range of concentrations, LB medium was used in this work, but certainly RM medium could further improve the output signal at the lower detection limit.

Response assay of all biosensors with time

Cells from exponential-phase were treated with 5 µM of lead, and the fluorescence was measured at different time points (**Fig. 4**). The fluorescence intensity of all six biosensors increased with induction time. An induction period of 8 hours gave sufficient and stable fluorescence signals when cell growth just entered the stationary phase.

Evaluation of the lead whole-cell biosensors

The dose response curves of these whole-cell biosensors toward lead at different concentrations were shown in **Fig. 5**. The green fluorescence emitted by the bacteria went up as the concentration of lead increased. However, it only increased when lead concentration was below 50 µM and decreased gradually at higher concentrations. The reduction in fluorescence was probably attributed to impaired cell growth at high concentrations of lead. The maximum fluorescence intensity of S21 was 18.7 times

of the background level, and 15.6 times and 16.3 times respectively for S22 and S23. Both S21 and S23 after re-configuring the physical structure of the *pbr* operon performed better in the strength of the output signal compared with S22 the one with native configuration.

The inducer independent LuxR variant and the promoter it regulates from the quorum sensing operon has been developed to serve as a signal amplifier in gene circuit design. The output signal was further enhanced by incorporating the positive feedback circuit. The maximum fluorescence intensity of D41 was 26.6 times of background, which was 1.42 times of the corresponding design without positive feedback, S21. Similarly, D42 gave the maximum signal 30.3 times of the background, which was 1.94 times of the one without positive feedback (S22); and the output signal from D43 was 24.2 times of the background and 1.48 times of S23. In all three configurations, the addition of the positive feedback loop significantly increased the strength of the signal and this demonstrated the role of positive feedback as signal amplifier. Some other effect of positive feedback circuits include enhanced sensitivity, memory, and increased noise level (Demongeot *et al.*, 2000, Hornung & Barkai, 2008, Chang *et al.*, 2010). No significant difference in sensitivity due to positive feedback circuit was observed in this study. Interestingly, the sensitivity of biosensors without positive feedback is either the same or slighter better than that of biosensors with positive feedback. Positive feedback is known to be able to increase the sensitivity and/or amplify the output signal, but the extent of the two effects could be different depending on the genetic context. Still, it is unexpected that the genetic circuits with positive feedback are less sensitive in this study. One possible reason is that the divergent promoter in the *pbr* operon is very short, and the distance from the start codon of the first gene on both sides is only 86 bp. The activation of transcription from this promoter is associated with a distortion of local DNA caused by the conformational change of PbrR dimer upon the binding of lead

ion (Parkhill *et al.*, 1993, Outten *et al.*, 1999). Therefore, replacing genes next to the promoter may affect the local structure of the DNA. In this study, the genes *pbrABCD* are replaced by *gfp* in the biosensors without positive feedback and replaced by *luxR* in the biosensors with positive feedback. The effect of *gfp* and *luxR* DNA on the expression from this promoter could be different, which may be the reason why biosensors without positive feedback have slightly better sensitivity. This study provides one more evidence that genetic context plays a role in the performance of the genetic circuit. Although the effect of positive feedback circuit on the performance of the biosensors may be different depending on the specific genetic regulatory elements, it is certainly a useful design strategy that should be taken into account if the aim is to increase the sensitivity or output signal, as demonstrated in many cases.

The memory and noise propagation are also important features for studying circuit design and engineering, but were not tested in this study, since as whole-cell lead biosensors, the designed circuits would be used at the population level for quick one-time detection. In this sense, single-cell level signal variance or memory is not directly relevant to the purpose of the designed circuit.

As shown in **Fig. 5**, all six biosensors were slightly leaky, ranging from 1.6-2.8 times of signal from the DH5α negative control. Moreover, all six designs were able to detect lead at a concentration as low as 0.01 μM. According to the standards for drinking water quality, lead content should not exceed 0.01 mg L^{-1}, which is 0.048 μM. This is within the detection limit of our designs. Moreover, S23, the one with *pbrR* and *gfp* under the same promoter, demonstrated even higher sensitivity and it emitted fluorescence about 4 times of the background level in response to lead at a concentration of 0.001 μM. The results showed the importance of configurations in the design of biosensor gene circuits with the same regulatory elements. Since transcription in opposite directions from a divergent promoter

could be differentially regulated, it is not surprising that when *gfp* is placed on the same side of the promoter as *pbrR* (S23), the circuit is more sensitive to lead, compared with the designs with *gfp* being transcribed from the *pbrABCD* side of the promoter (S21 and S22). Collectively, the results suggest that in the native *pbr* operon, the initial uptake of lead into the cell activates the transcription of *pbrRT* at very low concentration of lead. As a result, the expressed PbrT transports more lead into the cells. When accumulated at a relatively higher concentration, the increased level of lead bound PbrR activates expression of the genes *pbrABCD,* which leads to the export of lead to avoid toxic effects due to lead accumulation in the cell. This discovery will contribute to the understanding of the molecular mechanism of the lead resistance operon *pbr* and provide insights to the design of other heavy metal biosensors.

Overall, the two strategies we employed in this work both improve the performance of the lead biosensors based on the regulatory elements from the *pbr* operon. This study not only develops whole-cell lead biosensors that can detect lead below the action level in drinking water, but also highlights the importance of configuration and positive feedback loops in improving the sensitivity and output signal strength, which will provide insights for designing whole-cell biosensors in future.

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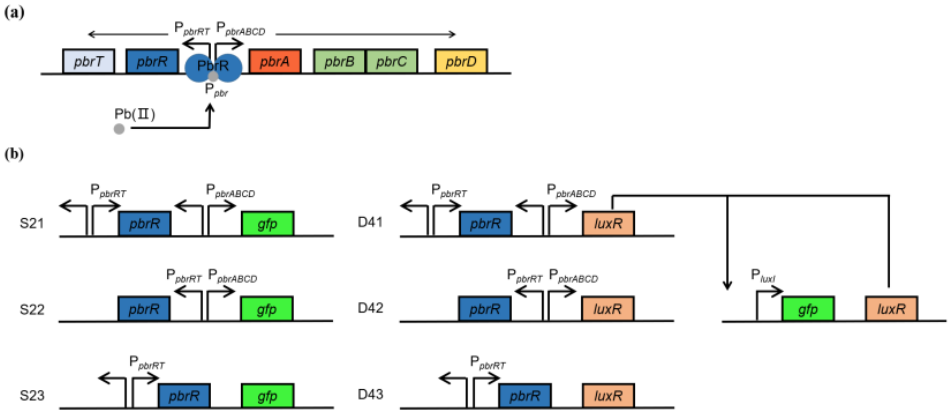

Fig. 1 (a) Schematic representation of the *pbr* operon in *Cupriavidus metallidurans* CH34. PbrR forms a dimer and binds to the divergent promoter of the operon (P_{pbrRT}-$P_{pbrABCD}$). In presence of lead, the PbrR-lead complex goes through a conformational change and activates the transcription of the genes on both sides. **(b)** Genetic circuit design of lead biosensors in this study. S21: the transcription of *pbrR* and *gfp* are controlled by the promoter P_{pbrRT} and $P_{pbrABCD}$, respectively, but they are expressed from separate operons; S22: *pbrR* and *gfp* are expressed from opposite sides of the divergent promoter in a way similar to the native operon with *gfp* replacing *pbrABCD*; S23: *pbrR* and *gfp* are expressed from the same side of the promoter P_{pbrRT}; D21: the configuration is similar to S21 but a positive feedback loop is introduced using the LuxR activator and its cognate promoter P_{luxI}; D22: it has the divergent configuration similar to S22 with positive feedback loop; D23: It is similar to S23 with positive feedback.

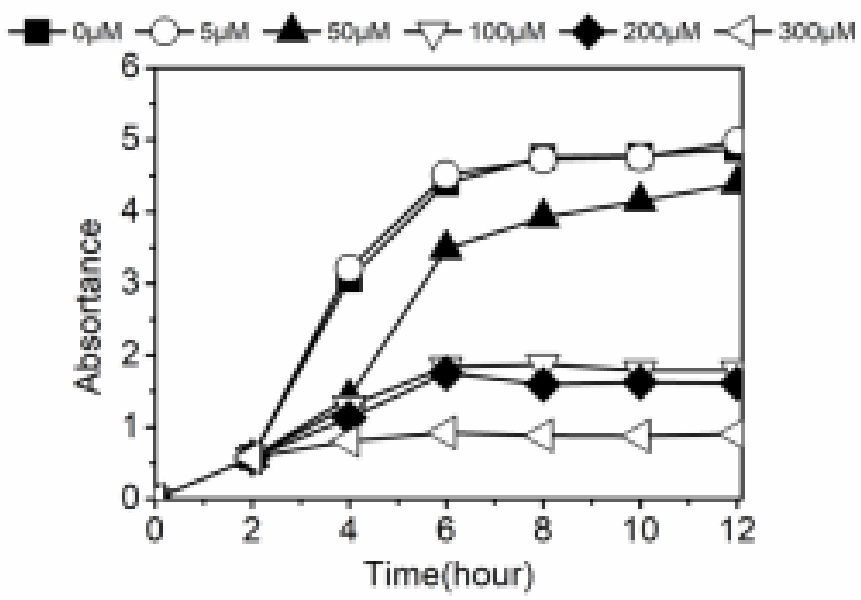

Fig. 2 Growth curves of *E. coli* DH5α with lead concentration ranging from 0 to 300 μM.

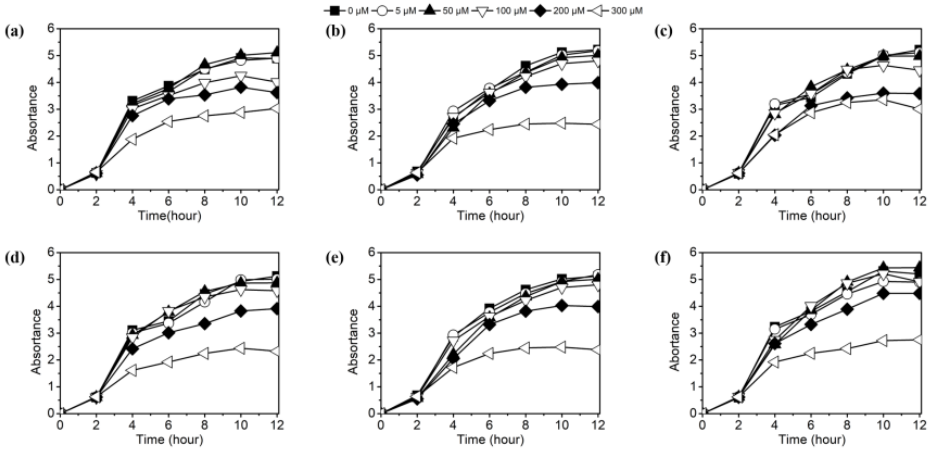

Fig. 3 Growth curves of the six lead biosensors with lead concentration ranging from 0 to 300 μM. **(a)** biosensor S21; **(b)** biosensor S22; **(c)** biosensor S23; **(d)** biosensor D41; **(e)** biosensor D42; **(f)** biosensor D43.

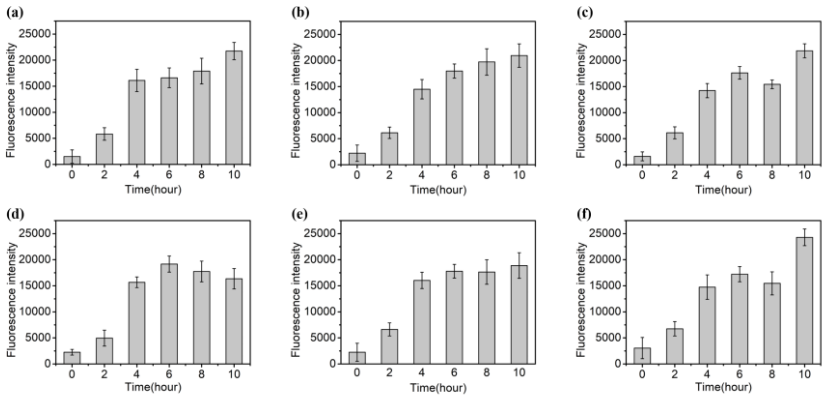

Fig. 4 Time-response assay of the six lead biosensors with 5 μM of lead. Fluorescence intensity is normalized to the OD_{600} of the culture. **(a)** biosensor S21; **(b)** biosensor S22; **(c)** biosensor S23; **(d)** biosensor D41; **(e)** biosensor D42; **(f)** biosensor D43.

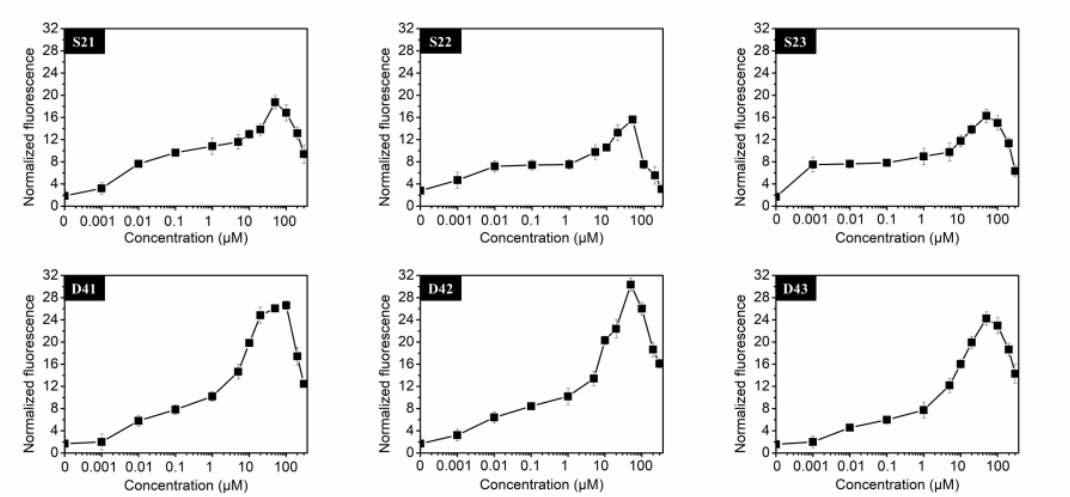

Fig. 5 Dose-response assay of the six lead biosensors. All biosensors were exposed to lead nitrate at different concentrations (0, 0.001, 0.01, 0.1, 1, 5, 10, 20, 50, 100, 200 and 300 μM). Fluorescence is normalized to the background fluorescence of the negative control *E. coli* DH5α. **(a)** biosensor S21; **(b)** biosensor S22; **(c)** biosensor S23; **(d)** biosensor D41; **(e)** biosensor D42; **(f)** biosensor D43.

Table 1. Plasmids and strains used in this study.

Plasmid/Strain	Relevant characteristic	Reference/Source
Plasmids		
pUC57-G7-Kan	*kan ΔpbrA*-P_{pbrRT}-*pbrR-ter-ΔpbrR*-$P_{pbrABCD}$	Synthesized by GENEWIZ
pUC57-gfp-Kan	*kan gfp*	Synthesized by GENEWIZ
pET28a	*kan* T7 *pro*-MCS- T7 *ter*	Invitrogen
pGN12	*kan* $P_{LtetO-1}$-*luxRΔ*$_{2-162}$	Nistala *et al*., 2010
pGN68	*cm* P_{luxI}-*gfp-luxRΔ*$_{2-162}$	Nistala *et al*., 2010
pABG	*kan ΔpbrA*-P_{pbrRT}-*pbrR-ter-ΔpbrR*-$P_{pbrABCD}$-*gfp*	This study
pCG	*kan ter-pbrR*-$P_{pbrABCD}$-*gfp*	This study
pDG	*kan ΔpbrA*-P_{pbrRT}-*pbrR-gfp*	This study
pABR	*kan ΔpbrA*-P_{pbrRT}-*pbrR-ter-ΔpbrR*-$P_{pbrABCD}$-*luxRΔ*$_{2-162}$	This study
pCR	*kan ter-pbrR*-$P_{pbrABCD}$-*luxRΔ*$_{2-162}$	This study
pDR	*kan ΔpbrA*-P_{pbrRT}-*pbrR-luxRΔ*$_{2-162}$	This study
Strains		
E.coli DH5α	F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR17*($r_K^-m_K^+$), λ^-	Transgen Biotech
S21	*E.coli* DH5α harboring pABG	This study
S22	*E.coli* DH5α harboring pCG	This study
S23	*E.coli* DH5α harboring pDG	This study
D41	*E.coli* DH5α harboring pABR and pGN68	This study
D42	*E.coli* DH5α harboring pCR and pGN68	This study
D43	*E.coli* DH5α harboring pDR and pGN68	This study