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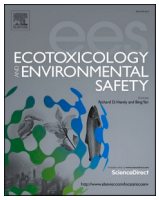

Monitoring arsenic using genetically encoded biosensors *in vitro*: The role of evolved regulatory genes

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ABSTRACT

Toxic pollutant (TP) detection *in situ* using analytical instruments or whole-cell biosensors is inconvenient. Designing and developing genetically coded biosensors *in vitro* for real-world TP detection is a promising alternative. However, because the bioactivity and stability of some key biomolecules are weakened *in vitro*, the response and regulation of reporter protein become difficult. Here, we established a genetically encoded biosensor *in vitro* with an arsenical resistance operon repressor (ArsR) and *GFP* reporter gene. Given that the wildtype ArsR did not respond to arsenic and activate GFP expression *in vitro*, we found, after screening, an evolved ArsR mutant ep3 could respond to arsenic and exhibited an approximately 3.4-fold fluorescence increase. Arsenic induced expression of both wildtype ArsR and ep3 mutant *in vitro*, however, only ep3 mutant regulated the expression of reporter gene. Furthermore, the effects of cell extracts, temperature, pH, incubation, and equilibrium time were investigated, and the equilibration of reaction mixtures for 30 min at 37 °C was found to be essential for *in vitro* arsenic detection prior to treatment with arsenic. Based on our data, we established a standard procedure for arsenic detection *in vitro*. Our results will facilitate the practical application of genetically encoded biosensors in TP monitoring.

1. Introduction

Toxic pollutant (TP) such as heavy metal ions, persistent organic pollutants, and biotoxins are a worldwide environmental problem threatening human health and ecosystem (Bereza-Malcolm et al., 2015; Kim et al., 2018). Detection and monitoring of TP are extremely important for preventing the occurrence of TP poisoning. Traditionally, TP detection and monitoring have been performed using expensive, laborious, and time-consuming equipment, and the procedures could only be carried out in laboratory with specially trained professionals. Whole-cell biosensors containing genetically encoded genes are a promising alternative analytical tool, having been used in environmental monitoring, cell factory, and disease diagnosis (Gui et al., 2017). Compared to traditional analytics, whole-cell biosensor assay can be rapidly and cost-effectively performed with cheap instruments (Bereza-Malcolm et al., 2015; Jia et al., 2019). However, both analytical instruments and whole-cell biosensors are inconvenient for TP detection *in situ*. Therefore, cell-free protein synthesis systems can be combined with genetically encoded biosensors to develop a simple and feasible technology for real-world environmental monitoring (Soltani et al., 2018).

Cell-free protein synthesis is a common biotechnology tool in protein studies (Kigawa et al., 2004; Sun et al., 2013; Garamella et al., 2016), overcoming the limitations associated with cell wall and membrane, and provides a simpler, flexible, and better-defined context for engineering (Karig et al., 2012), having the advantage of rapid expression, easy regulation, and less interference. The potential application of cell-free protein synthesis in environmental monitoring is attractive (Karig, 2017; Soltani et al., 2018). While most cell-free expression systems focus

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on maximizing the yield of proteins and metabolites (Sawasaki et al., 2002; Kim et al., 2006; Carlson et al., 2012), detection of TPs aims at elaborately regulating the expression of reporter genes *in vitro* by regulatory genes in TP-responsive operons. Using a 3OC12-HSL-responsive operon, Wen et al. designed a cell-free biosensor for detecting quorum sensing molecules in respiratory samples from Pseudomonas aeruginosa-infected patients (Wen et al., 2017). In addition, the cell-free transcription-translation system was applied in an established mercury-responsive biosensor *in vitro* (Didovyk et al., 2017; Lopreside et al., 2019). However, success stories regarding TP detection with genetically encoded biosensors *in vitro* are limited. Besides the expression of regulatory proteins and reporter proteins, the interaction of regulatory proteins with DNA binding sites and analytes is challenging *in vitro*. Additionally, the bioactivity and stability of some regulatory proteins is greatly impacted *in vitro*, causing failure in TP detection.

In the present study, to develop a convenient analysis method for environmental monitoring, we intended to employ *in vitro* protein expression technology to detect TPs based on evolved genetically encoded biosensors. Arsenic is a worldwide typical toxic pollutant, and arsenic-responsive biosensors have been applied in real-world detection (Siegfried et al., 2012; Kaur et al., 2015). For the establishment of an arsenic-responsive biosensor *in vitro*, we investigated several evolved mutants of arsenic-resistance regulatory proteins and found that the ep3 mutant of arsenic-resistance (ArsR) is most sensitive to arsenic *in vitro*. To elucidate the relationship of regulatory proteins to reporter proteins, we investigated the expression of these two proteins using western blotting. Further, to improve the sensitivity of biosensors, we surveyed the effects of cell extracts, temperature, pH, incubation, and equilibrium time on detection. With optimized reaction conditions, we established a sensitive, portable, and easy-to-use technique for environmental monitoring based on genetically encoded biosensors, which will facilitate the application of biosensors in real-world TP detection.

2. Materials and methods

2.1. Strains and media

Escherichia coli (*E. coli*) strain DH5α was used for arsenic detection experiments *in vivo* and crude cell extract preparation. *E. coli* strain Rosetta was used for all the other experiments. All bacteria cultures were grown at 37 °C with constant shaking at 200 rpm. 2xYT + P medium was supplemented with 34 μg/mL chloramphenicol (Cm) (2xYT + P–Cm). TB medium was supplemented with 200 μg/mL ampicillin (TB-amp). Solid media were prepared adding 15 g/L agar.

2.2. Plasmid construction

PUC18-Ars-GFP (including wildtype and ArsR mutants) were constructed as described (Li et al., 2015). Since the primary antibody of the ArsR protein was not available, ArsR genes in pUC18-ArsR-GFP were replaced by the his-ArsR chimeric gene using the overlap polymerase chain reaction (PCR) technique to detect the expression of ArsR proteins *in vitro*. The overlap PCR involves three separate PCR systems that mix the two DNA fragments produced in the first stage reaction to form a template for the second stage. First stage PCR primers (Table S1) were: flanking primer I (ArsR-BamHI-F), hybrid primer I (His-RBS-R), hybrid primer II (His-GS-wt-F or His-GS-ep3-F), flanking primer II (pUC18-XhoI-R), and two DNA fragments with overlapping sequences. When the two fragments are mixed, they can anneal, using the original two flanking primers, participating in the second stage PCR to produce the final DNA fragment. After digestion with *Hind*III-HF and *Xho*I (New England Bio labs), the PCR products were cloned into the pUC18-GFP plasmid, resulting in pUC18-his-wt-GFP and pUC18-his-ep3-GFP.

2.3. Biosensor induction in Vivo

Mutant or wildtype arsenic-responsive bacterial biosensor was grown overnight in a 3 mL TB-amp. The arsenic-induced expression assay was prepared using 1:1 mixture of 1 mL arsenic standard solution with 1 mL of 1:50 dilution of overnight cell culture and incubated at 37 °C for 1 h with shaking at 200 rpm. Cell cultures were collected, washed, and resuspended with chilled and sterilized phosphate-buffered saline (PBS). GFP fluorescence was measured using a SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) and flow cytometer (BD FACS AriaIII, BD Biosciences).

2.4. Biosensor induction in Vitro

Commercial *E. coli* S30 Extract System for circular DNA was purchased from Promega (Southampton UK). Each reaction was prepared following the manufacturer's guidelines up to a final volume of 50 μL containing 1 μg pUC18-ep3-GFP or pUC18-wt-GFP plasmid DNA (Li et al., 2015). For characterization assays, after 30 min of reaction equilibrium, 0.5 μL arsenic standard solution in distilled deionized water (ddH_2O) was added as an inducer (within the 50 μL total reaction volume) to a final concentration of 50 μg/L. Reactions were incubated at 37 °C for 2 h. The reactions were then transferred to a white 96-well plate (CORNING) with clear flat bottom. Absorbance was measured using the SpectraMax i3x Multi-Mode Microplate Reader, and the fluorescence (excitation at 485 nm, emission at 520 nm) was measured.

Homemade cell extract reactions were prepared with 20 μL cell extract, 40 μL energy solution, 10 μL amino acid mixtures, 2 μg of pUC18-ep3-GFP plasmid DNA, and nuclease-free water up to a final volume of 100 μL. After 30 min of reaction equilibrium, 1 μL arsenic standard solution in ddH_2O was added as inducer. After incubation for the designated time, GFP measurements were performed following the same method described above for commercial reactions.

2.5. Cell extracts

Cell extracts were prepared for *E. coli* strains DH5α, BL21, or Rosetta using the protocol described below (Sun et al., 2013; Kwon and Jewett, 2015). Briefly, the overnight culture was inoculated into 50 mL 2xYT + P–Cm medium with 1:20 dilution and grown for 4 h at 37 °C. The culture with 1:20 dilution was added to 500 mL 2xYT + P–Cm medium and incubated for 6 h at 37 °C. The larger culture was centrifuged at 4 °C to pellet cells. The supernatant was discarded, and the pellet was re-suspended in S30 A buffer two times. Pellet cells were frozen overnight at −80 °C. The next day, the pellets were re-suspended on ice with cold S30A buffer. The mixtures were subsequently sonicated on ice. After sonication, the mixtures were centrifuge at 12,000×*g* for 15 min at 4 °C. The supernatant was gently transferred to new tubes and complemented with dithiothreitol (DTT). After incubation, supernatants were collected and dialyzed using a dialysis tube (D-Tube™ Dialyzer Maxi, MWCO 6–8 kDa, Thermo-Scientific) at 4 °C for 3 h. Finally, the dialyzed extracts were centrifuged, and the supernatants (cell extracts) were collected into fresh tubes. The extracts were flash-frozen in liquid nitrogen before storage at −80 °C until needed.

Energy solution was prepared with 700 mM HEPES (pH 8.0), 21 mM ATP, 21 mM GTP, 12.6 mM CTP, 12.6 mM UTP, 2.8 mg/mL tRNA, 3.64 mM CoA, 4.62 mM NAD, 10.5 mM cAMP, 0.95 mM folinic acid, 14 mM spermidine, and 420 mM 3-PGA. The amino acid mixtures were 50 mM of 20 amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, proline, serine, threonine, valine, tryptophan, phenylalanine, and tyrosine).

2.6. Western blot

Once the S30 extract reaction was completed, ep3 and GFP proteins

were collected following manufacturer's instructions (Promega, Southampton UK), and ep3 proteins were fractionated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride (PVDF) membranes (Roche, Switzerland), and immunoblotted with antibodies anti-his (1:1000, ZENBIO, China) and anti-GFP (1:1000, Cell Signaling Technology, USA). The protein bands were visualized using ECL reagent (Boster, China).

2.7. Data analysis and statistics

The flow cytometry data were analyzed using the FlowJo software (v.10.0). Other statistical analyses were performed using GraphPad Prism software (GraphPad, California US). Data are shown as mean and standard deviation (n = 3 biologically independent samples). An independent *t*-test or one-way analysis of variance (ANOVA) test was used to analyze the experimental data. A p-value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Screening of regulatory gene mutants for TP detection in vitro

Regulatory proteins are the core of genetically encoded biosensors and control biosensor response to analytes as a switch. In our previous study, different mutants of arsenic regulatory proteins were screened from the ArsR mutation library by directed evolution (Li et al., 2015). To obtain the biosensor perfectly suited for arsenic detection *in vitro*, biosensors were transformed into *E. coli* DH5α, and the response of these mutants to arsenic was measured using microplate readers and flow cytometer *in vivo*. Fig. 1a and b shows that the response to arsenic of the ArsR mutant ep3 significantly increased by approximately 8-fold, which was significantly higher than that of other mutants. A commercial *E. coli* cell-free reaction kit was used to detect arsenic-responsive GFP expression *in vitro*. As shown in Fig. 1c, most of mutants and wildtype ArsR failed to regulate GFP expression *in vitro*. Only the mutant ep3 exhibited a notable increase in fluorescence response to arsenic compared to the control group, which was consistent with *in vivo* experiments. These data show that the ArsR mutant ep3 was more sensitive and suited for *in vitro* arsenic detection than wildtype ArsR. These results suggest that the performance of regulatory proteins is very important for TP detection, and directed evolution is an effective tool to improve the sensitivity of genetically encoded biosensors *in vitro*.

3.2. Regulation of reporter gene GFP expression by arsenic-responsive protein ArsR in vitro

For *in vitro* TPs detection, regulatory proteins must respond to analytes and switch on the expression of reporter proteins *in vitro*. Fig. 2a shows that the regulatory protein P1 controls expression of the reporter protein P2. The response of P1 to analyst and elaborately regulating the expression of P2 is essential for the performance of genetically encoded biosensors. To discover the key points of expression regulation *in vitro*, we employed his-tag fused-ArsR biosensors. *In vivo* and *in vitro* experiments demonstrate that his-tag did not affect the response of biosensors to arsenic (Fig. S1). We investigated the expression of regulatory genes and reporter genes. Fig. 2b shows that arsenic induced the expression of both wildtype and ep3 ArsR, however, increased wildtype ArsR levels did not significantly upregulate the expression of GFP as ep3 did. Ep3 exhibited superior performance for TP detection. Therefore, these results imply that the interactions between regulatory proteins and TP may be impacted by *in vitro* action condition, and confirm that robust regulatory proteins are a key factor for *in vitro* TP detection with genetically encoded biosensors.

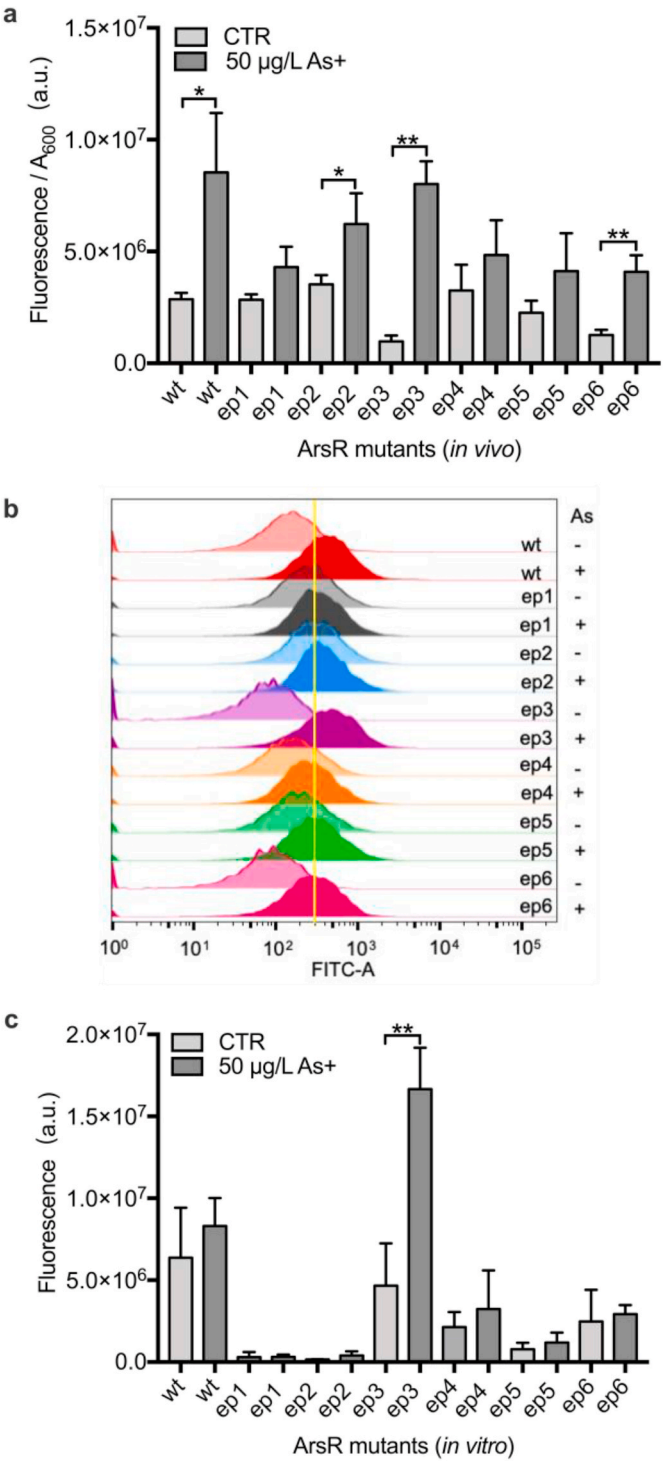

Fig. 1. Response of ArsR mutants to arsenic *in vivo* and *in vitro*. After induction with 50 μg/L arsenic standard solution, the responses of whole-cell biosensors (*in vivo*) with different ArsR mutants were detected by microplate reader (a) and flow cytometer (b), and the responses of cell-free biosensors (*in vitro*) with different ArsR mutants were detected by microplate reader (c). Values are mean ± s.d. (n = 3). $p < 0.05$ (*), $p < 0.01$ (**).

3.3. Effects of crude cell extract on biosensors in vitro

To develop a simple, economical, and practical method for cell extract preparation, the activity of cell extracts derived from three *E. coli* strains and cell disruption methods (Sun et al., 2013; Kwon and Jewett, 2015) were investigated. We used a gradient concentration arsenic

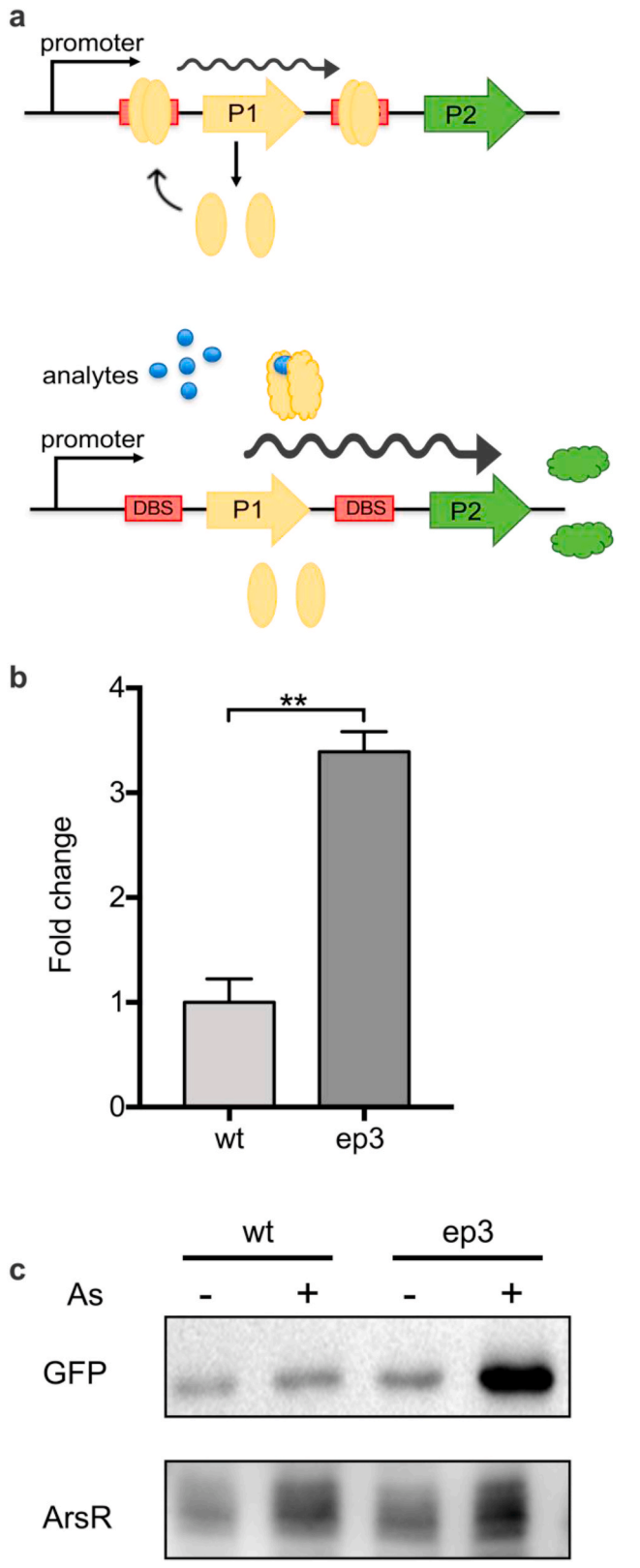

Fig. 2. Regulating GFP expression with arsenic-responsive proteins *in vitro*. (a) Schematic diagram of regulated expression in genetically encoded biosensors. (b) After induction with 50 μg/L arsenic standard solution, the induction of ep3 biosensor was compared with wildtype biosensor with commercial *E. coli* cell-free reactions. Values are mean ± s.d. (n = 3). $p < 0.01$ (**) (c) The expression of ArsR and GFP proteins were detected by Western blot.

standard solution to reveal the effects of different bacterial extracts on response of biosensors (Fig. 3a). There was slight response to arsenic in the groups of DH5α and BL21 cell extracts, while a dose-dependent GFP output was observed in Rosetta cell extracts. Moreover, we compared different cell disruption methods on biosensor response, including sonication alone and sonication combined with liquid nitrogen freeze-thaw. Fig. 3b shows that there was no significant different response between the group of biosensors in the sonication alone and sonication plus freeze-thaw groups ($p>0.05$). Since sonication alone is simple and time-saving, these results clearly indicate that cell extracts derived from Rosetta *E. coli* strains prepared with sonication are suitable for arsenic detection with genetically encoded biosensors *in vitro*.

3.4. Reaction conditions of arsenic-responsive biosensor in vitro

To detect the output of the reporter protein sensitively and quickly, we optimized the reaction conditions. First, the optimal reaction temperature of biosensor response was investigated. As shown in Fig. 4a the levels of GFP expression were different at 20, 30, 37, and 40 °C. Notably, GFP expression at 37 °C was significantly higher than that at other temperatures. Second, the effect of pH on biosensor response was explored. The reaction system was mainly constituted with crude cell extracts, energy solution, and amino acid mixtures. The pH value of the three components was different, thereby affecting the pH of the reaction system. Thus, the pH of the reaction system needed to be readjusted. Fig. 4b shows that the arsenic-induced increase in GFP level was significantly higher at pH 7.2 than that at pH 6.0 and 8.0.

For unknown reasons, there was no significant biosensor response to arsenic when it was added to the reaction mixture at the beginning of the experiments. We equilibrated the reaction mixtures for different times at 37 °C and induced them with arsenic. A time-dependent response of the biosensors that peaked at 30 min was observed (Fig. 4c). In addition, we detected the output of GFP under different arsenic induction times. Fig. 4d shows that the expression of GFP increased with the extension of time and reached a peak at 4 h, and the fluorescence of the GFP expressed decreased overnight. The GFP fluorescence change was found to be 2-fold at 2 h, which was clearly higher than that at other time points. These results indicate that the optimal detection condition for this system is equilibration for 30 min, followed by induction at 37 °C for 2 h at pH 7.2.

3.5. Dose-dependent response of biosensors to arsenic in vitro

According to the data above, a standard procedure for arsenic detection was developed. Briefly, cell extracts, energy solution, amino acids, and arsenic-responsive biosensor plasmids were mixed together and equilibrated for 30 min at 37 °C. Then, the samples with arsenic were added into reaction system and incubated at 37 °C for 2 h, and the GFP fluorescence was detected using a microplate reader (Fig. 5a). Using this protocol, the dose response of the ep3 mutant to arsenic was investigated. As shown in Fig. 5b, a good linear response of the biosensor to arsenic was observed between 0 and 50 μg/L, which was important to obtain a standard response curve. The lower right panel of Fig. 5b showed that the detection limit (LOD) of arsenic was 3.65 μg/L. The LOD is calculated according to:$LOD=3\sigma/S$.Where S is the standard deviation (SD) of the blank and σ is the slope of linear (Shrivastava, 2011). These results indicate that genetically encoded biosensors can be used for quantitative arsenic detection *in vitro*.

4. Discussion

TP-induced promoters and reporter genes have been successfully used in whole cell biosensors (Rogers, 2006; Gudlavalleti et al., 2017; Alhadrami, 2018). To accelerate the application of genetically coded biosensors in the real-world TP monitoring, technologies in molecular, synthetic and computational biology have been employing to improve

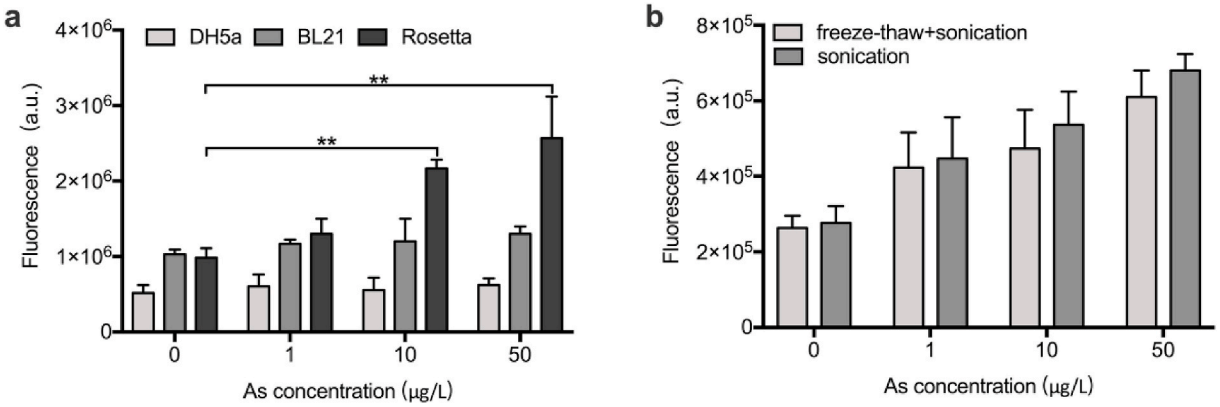

Fig. 3. Optimization of the crude cell extract of cell-free expression system. (a) The activity of cell-free extracts of three different strains. (b) Effect of different cell disruption methods on crude cell extract activity in reaction system. Values are mean ± s.d. (n = 3). $p < 0.05$ (*), $p < 0.01$ (**).

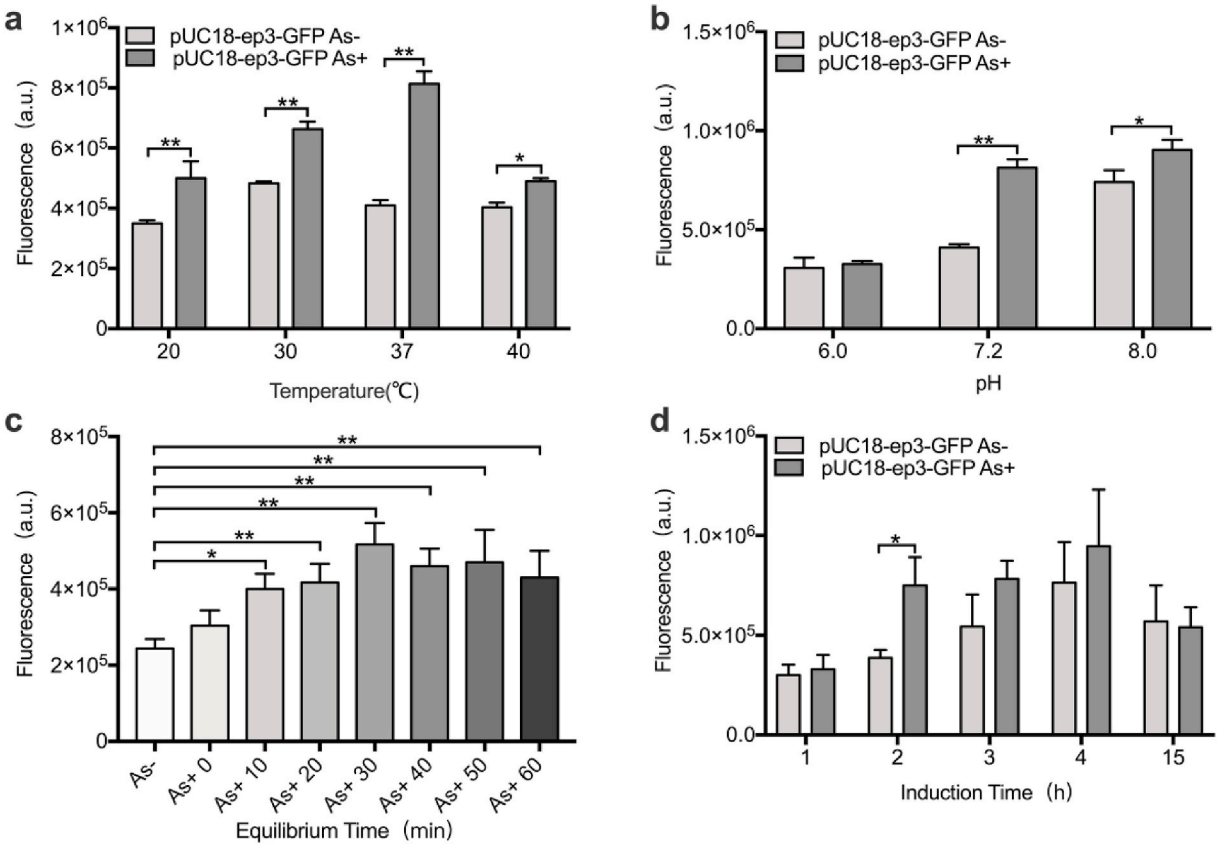

Fig. 4. Reaction conditions of cell-free arsenic biosensors. Values are mean ± s.d. (n = 3). $p < 0.05$ (*), $p < 0.01$ (**). (a) Effect of temperature on TX-TL cell-free Reaction. (b) The optimal reaction pH for cell-free expression. (c) Effect of equilibrium time on arsenic detection. (d) Time-Dependent Response to arsenic.

the performance of these biosensors, such as promoter and protein engineer, transcriptional and translational regulation, reporter gene assay. By placing auxiliary ArsR binding site and tuning the length of the 5′-untranslated region, van der Meer and his colleagues established an ArsR-operator system with a very good signal-to-noise ratio, which was the extreme sensitivity to micrograms per liter arsenite concentrations (Stocker et al., 2004; Merulla and van der Meer, 2016). With a modular cascaded signal amplifying methodology, Wan X. et al. improved the sensitivity of arsenic whole-cell sensor (LOD ≤0.1 ppb) (Wan et al., 2019). In previous study, we improved the detection limit with an ArsR mutant isolated from an error-PCR library with high-throughput screening (LOD ≤0.75 μg/L) (Li et al., 2015). These efforts are helpful to advance the practical applications of arsenic biosensors. However, TP monitoring with bacteria is inconvenient, because it is difficult to maintain cell viability and stability outside laboratory conditions. A few research group have tried to developed cell-free biosensors to detect mercury (Pellinen et al., 2004; Lopreside et al., 2019), antibiotics (Duyen et al., 2017) and quorum sensing molecules (Wen et al., 2017). However, the number of documented cell-free biosensors are much less than whole-cell biosensors. The lack of high-performance regulatory proteins and deep understanding of reaction conditions prevents the application of genetically-encoded biosensors *in vitro*. In this study, with an evolved mutant, we established an experimental procedure to detect arsenic with cell-free biosensors. The detection limit of this cell-free biosensors is 3.65 μg/L (Fig. 4b), which have met the demands of practical applications (the WHO/EPA/China safety limit level: 10 μg/L).

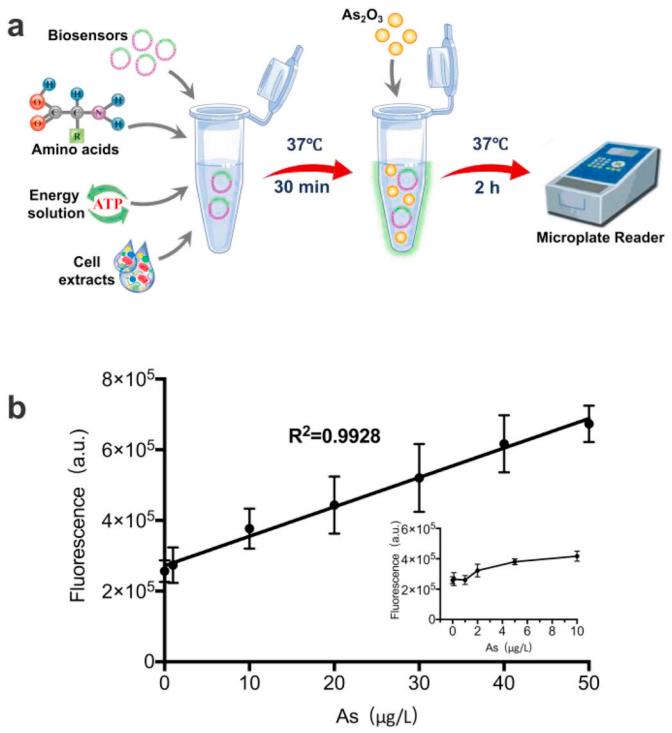

Fig. 5. Arsenic detection with genetically encoded biosensors *in vitro*. (a) Schematic of arsenic induction reaction. (b) Dose-dependent response of biosensors to arsenic *in vitro*. R^2 values result from linear interpolation of the averages from three replicates (R^2 = 0.9928). Concentration calculated from hyperbolic slope.

Protein synthesis *in vitro* has been developed over five decades (Chong, 2014). For the advantage of high controllability, it is applied to the fields of bioenergy production, drug synthesis, bioremediation and protein engineering (Karig et al., 2012; Caschera et al., 2018). Well-folded and highly-active proteins *in vitro* are the perfect candidates for this protein synthesis system. For *in vitro* TP detection, two types of proteins (regulatory protein and reporter protein) are involved, and regulatory proteins must respond to analytes and switch on the expression of reporter proteins *in vitro* (Fig. 2a). In our study, we found that an evolved ArsR mutant ep3 could effectively respond to arsenic and regulate GFP expression (Fig. 1). Wildtype ArsR proteins could be well-expressed *in vitro*, however, they could not regulate the expression of GFP (Fig. 2b). We hypothesized that some of wildtype ArsR proteins could not be folded correctly *in vitro*, which affect the activation of biosensors. These results suggest that improving the performance of regulatory proteins is important for establishing a genetically encoded biosensor *in vitro*, and directed evolution is a useful tool in this field. The reaction conditions are very important for arsenic detection *in vitro*.

The reaction conditions are very important for arsenic detection *in vitro*. In present study, we found if arsenic was added to the reaction system at the beginning of the experiments, the biosensors showed significant response. When reaction mixtures were equilibrated for 30 min before arsenic induction, the GFP fluorescence was significantly enhanced (Fig. 4c). The reason why reaction equilibrium time has a critical influence is not clear, and the phenomenon has not been documented in studies on genetically encoded biosensors, either *in vivo* or *in vitro*. We suspected that some ingredients of reaction mixtures might interact with arsenic, thereby preventing interaction of the arsR protein with arsenic. During equilibrium, these ingredients are degraded or blocked by others. As described by Karzbrun (Karzbrun et al., 2011), the fundamental kinetics of cell-free biosensors differs from that of whole-cell biosensors, which leads to a different performance of cell-free biosensors in quantitively detection than that of whole-cell biosensors (Chappell et al., 2013). To optimize the detection performance of biosensors better, the mechanisms of cell-free biosensors are worth being further explored.

In summary, we developed an *in vitro* genetically encoded biosensor technology for environmental monitoring by improving the performance of regulatory proteins and equilibrating reaction mixtures. This study will facilitate the practical application of genetically encoded biosensors *in vitro*.

Author contributions

Xuanyu Wang, Methodology, Investigation, Writing - original draft. Kaili Zhu, Investigation, Data curation. Dongdong Chen, Investigation, Data curation. Juan Wang, Investigation, Data curation. Xiaofei Wang, Validation, Funding acquisition. An Xu, Conceptualization, Supervision. Lijun Wu, Conceptualization, Supervision. Luzhi Li, Funding acquisition, Project administration, Supervision, Writing - review & editing. Shaopeng Chen, Funding acquisition, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111273.

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