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Evolved bacterial biosensor for arsenite detection in environmental water

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Running title: Arsenite detection with evolved bacterial biosensor

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ABSTRACT

Arsenic, a ubiquitous presence in the biosphere, often occurs from both natural and anthropogenic sources. Bacterial biosensors based on genetically engineered bacteria have promising applications in detecting the chemical compound and its toxicity. However, most of the bactiria biosensor takes advantage of the existing wild-type substrate-induced promoters, which are often low in specificity, affinity and sensitivity, and thus limiting their applications in commercial or field use. In this study, we developed an *in vivo* evolution procedure with bi-directional selection scheme for improving the sensitivity of arsenite-responsive bacterial biosensor through optimization of the inducible operon. As a proof of concept, we evolved the arsenite-induced arsR operon for both low background and high expression through three successive rounds of fluorescence activated cell sorting (FACS) with bi-directional strategy. An arsR operon variant with 12-fold higher activity over the control was isolated, confirming multiple rounds of construction and screening of mutation library, as described here, can be efficiently applied to bacterial biosensor optimization. The evolved arsenite-responsive biosensor demonstrated an excellent performance in the detection of low trace arsenite in environmental water. These results indicate that the technologies of directed evolution could be used to improve the performance of bacterial biosensors, which will be helpful in promoting the practical application of bacterial biosensors.

INTRODUCTION

Arsenic is a common contaminant throughout the environment in the air, water, soil and food, derived from both natural and anthropogenic sources.[1] It can enter human body through multiple pathways, such as drinking, eating, inhalation, and so on.[2] The acute arsenic poisoning can lead to vomiting, abdominal pain, diarrhoeanumbness and tingling of the extremities, muscle cramping and death in extreme cases.[3] Arsenic is also a dangerous environmental carcinogen, and chronic exposure to it is associated with the risk of cancer development.[4,5] Consequently, arsenic contamination tops the list of hazardous substances in Agency for Toxic Substances and Disease Registry (ATSDR) and the Environmental Protection Agency (EPA) in US for many years.[6]

Since most of arsenic contamination is natural origin and can never be eliminated permanently, rapid and cost-effective on-site analytical techniques for arsenic monitoring would assist in assessing arsenic pollution and preventing further arsenic exposure. The most commonly used arsenic detection analytics are the high-end chemical analytics, such as gas chromatography-mass spectroscopy (GC-MS),[7] high-performance liquid chromatography-mass spectrometry (HPLC-MS),[8,9] inductively coupled plasma-mass-spectrometry (ICP-MS),[10] etc. These traditional analytics are often expensive and can only be operated by experts, and thus preventing their applications on a regular basis. Meanwhile, scientists have been working to seek for alternative strategies for arsenic detection for years. As a promising alternative analytical tool, whole bacterial biosensor employs living microorganisms with genetic engineered sensing element to analyze the pollution risk.[11,12] Reported arsenite-induced bacterial biosensors utilize the regulatory machinery of the arsenite-responsive transcriptional repressor ArsR to control expression of reporter genes in response to arsenite exposure,[13,14] which directly take advantage

of the wild-type regulatory expression machinery. Since the whole arsenite sensing element needs a basal level of arsR expression to function, the genetic background could not be avoided. The basal genetic background will reduce sensitivity and detection limit of arsenite-responsive biosensors, thus limiting their applications in in-field detection.[15] Van der Meer and his colleagues successfully improved the sensitivity of arsenite-responsive biosensor with the method of two suppressor binding sites to decrease the background of reporter genes.[14] Herein, we described a method to obtain a more sensitive arsenite-responsive biosensor with directed evolution.

Gene expression engineering via directed evolution[16] has been extensively applied for the systematic improvement of antibody-binding affinity,[17] enzyme regulation,[18] constitutive promoter engineering[19] and microbial sensor improvement.[20] Here, we improved the sensitivity of an arsenite-responsive biosensor with high-throughput screening and directed evolution. Unlike the one directional screening strategy for the constitutive promoter or protein engineering, the evolution of a high sensitive arsenite-responsive biosensor aimed for both low background and high substrate-induced signal levels.

The purpose of this study is to obtain a more sensitive arsenite-responsive biosensor which could be applied for detecting arsenite contamination in natural water resources. To achieve this goal, we constructed a mutation library of arsenite sensing element with error-prone PCR, and screened for the mutants of low background and high expression level with a novel bi-directional selection system. After three successive rounds of directed evolution, an arsR operon variant with 12-fold higher activity over the wild-type was isolated, which exhibited a good performance in arsenite detection for the practical application.

MATERIALS AND METHODS

1. Strains and media

Escherichia coli (*E.coli*) strain DH5α was used for all experiments of constructing mutation libraries and FACS screening. *E.coli* strain TransI was used for all the other experiments. All bacteria cultures were grown at 37 °C (unless stated otherwise) with constant shaking at 180 rpm. TB media[21]was supplemented with 300 μg/mL Ampicillin to make media TB-amp. SOC and LB media were made as described previously.[22,23] Solid media was prepared by supplemented with 15 g/L agar.

2. Construction of Vector

DNA sequence of arsenite sensing elements in pPR-arsR-ABS[14] which derived from the *E.coli*plasmid R773,[24] including rrnB T1 terminators, ArsR DNA binding site (abs), ars promoter and ArsRrepressor gene, was amplified by PCR with primer pair PP1/PP2. The pPR-arsR-ABS plasmid was a friendly and generous gift from Dr. J.R. van der Meer (University of Lausanne, Switzerland). After digested by *Bgl*II-*Xho*I (New England Bio labs), the PCR products were cloned into pUC18 plasmid to substitute lac promoter. Then *GFP* gene was inserted down stream of arsenite operon to yield wild-type arsenite biosensor vector, pUC18-ars-gfp. The null variant plasmid, pUC18-AID-gfp, was constructed by replacing the arsenite sensing element with a non-functional *AID* gene fragment using *Hind*III-HF and *Xho*I (New England Bio labs). Primers are

listed in Table 1.The molecular construction path of pUC18-ars-gfp and pUC18-AID-gfp were provided with more details in the Supporting Information (Figure S1, Supporting Information).

Table 1. List of primers used in this study.

Primer Name	Sequence	PCR product	Restriction sites
PP1	AATTATGCAagatctGGGGCCGCAATTCCCAATTC	arsR-operon	*Bgl*II
PP2	CCTTTACTCATctcgagTTCCTCCTTATAAAGTTAATC	arsR-operon	*Xho*I
PP3	TATAAGGAGGAActcgagATGAGTAAAGGAGAAGAAC	GFP	*Xho*I
PP4	GCTCAGCTAATTatcgatTATTTGTATAGTTCATCCATG	GFP	*Cla*I
PP5	ATCGCCaagcttATGTATCCATATGATG	AID	*Hind*III
PP6	AAGCCGctcgagTCAAAATCCCAACATAC	AID	*Xho*I
EP1	ATTGGGGATCGGaagcttTCCAAG	arsR operon	*Hind*III
EP2	CTTTACTCATctcgagTTCCTCCT	arsR operon	*Xho*I

3. Biosensor induction

Each arsenite-responsive bacterial biosensor, with mutated or wild-type arsR operon, was grown overnight in 1 mL TB-amp. Bioreporter assay was prepared by 1:1 mixture of 1 mL arsenite standard solution with 1 mL diluted overnight cell culture, and incubated at 37 °C for 1 h with vigorous shaking (180 rpm shaker). Fully induced cells were pelleted and resuspended in chilled, sterilized PBS for further fluorescence check.

4. Directed evolution of arsR

4.1. Creating a diverse mutation library

The most commonly used method of error-prone PCR (ep-PCR) was employed to generating variants with random mutations. The primers EP1 and EP2 were used to amplify the 651 base pairs of the gene encoding arsenite sensing elements as the template. Ep-PCR reactions were performed using Taq DNA polymerase and the low mutation frequencies from 0.16 to 0.67 % (1.6 to 6.7 nucleotides per 1 kb) was achieved simply by varying the nucleotide ratio and the amount of $MnCl_2$ in the PCR reaction.[25] PCR products were ligated into *Hind*III-*Xho*I digested pUC18-AID-gfp and then transformed into DH5α electro-competent cells with MicroPulse electroporator (Biorad).[26] After shaked at 37 °C for 45 minutes, the transformation mixture was inoculated onto LB-agar plates with ampicillin and cultivated overnight. The colonies were collected directly from transformation plates and plasmid DNA was extracted with a standard plasmid-extraction kit (TransGen Biotech). The ideal screening library contained over 1,000,000 colonies.

4.2. Screening of the libraries

A special screening step for low background expression mutants was applied before the induction over-expression screening to form a novel bi-directional screening scheme. Briefly, a 1 mL aliquot of mutation library induced with or without arsenite was prepared as described above. In step I, all of the negative bacteria with low-background fluorescence were selected without substrate induction. In step II, after induction of arsenite, all the positive mutants with high fluorescence were sorted. In the step III and IV, the mutants were sorted as in the step I and II with slightly extreme gates. Finally, in the step V, the mutants with extremely high fluorescence were collected. In each step, the strains with wild-type gene and null mutant were prepared and worked as controls. Cells were resuspended and sorted for target mutants using a BD FACS AriaIII (BDBiosciences). After cell sorting in each step, the performance of mutants were

141 determined with flow cytometry and analyzed by BD FACSDiva software (BD Biosciences).
142 “Relative fluorescence intensity”, “folds of relative fluorescence” and “relative induction activity”
143 were used to quantify the performances of the mutants (Refer to Annotation II and Figure S2 in
144 the Supporting Information).

145

146 4.3. Mutation biosensor isolation and analyzing

147 Sorted mutants were plated on LB-amp plates. Clones were isolated, cultivated overnight and
148 induced as described above. The fluorescence response was analyzed with BD FACS AriaIII
149 (BD Biosciences). The mutants exhibiting desirable characteristics were re-transformed into
150 *E.coli*, and checked for induction performance. The final variants were subjected to sequence
151 analysis comparing with the wild-type gene.

152

153 5. Bacterial biosensor detection with unknown water sample.

154 Arsenite contaminated groundwater sample was collected in September 2014 from Togtoh
155 regions, Inner Mongolia. In order to monitor arsenite concentration of unknown water sample, an
156 arsenite calibration curves were prepared from commercially available arsenite stock solution of
157 1000 mg/L and a final induction concentrations of 0, 1, 2, 5, 10, 25 and 50 μg/L respectively was
158 established. The unknown water samples should be incubated in consistence with the calibration
159 samples, and the GFP fluorescence response value could thus give a predictional arsenite
160 concentration.

161

162 6. Flow cytometry and FACS.

A BD FACS AriaIII (BD Biosciences)）machine equipped with an argon laser was used for fluorescence measuring and cell sorting. BD Accudrop Beads (BD FACS™) were used to prepare the machine and set up for sorting. The GFP fluorescence of individual cells was detected with a 488-nm band of the excitation argon laser and registered in the “FITC” channel. The 70 nm nozzle tip was chosen for bacteria sorting. The system threshold rate was kept under 5,000 events/s and the bacteria were sorted in single cell mode. The BD FACSDiva software (BD Biosciences) was used for data acquisition and analysis.

7. Statistical analysis

All statistical analysis was performed using statistical software package OriginPro Version 8.6. All data were presented as means and standard derivations. The Student’s t test was used to assess the significance levels. A P-value of 0.05 or less was considered statistically significant.

RESULTS AND DISCUSSION

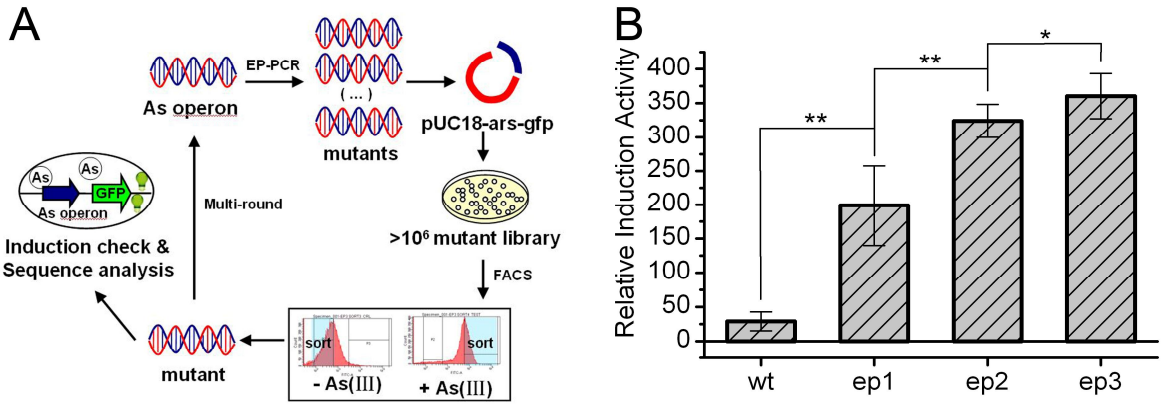

Figure 1. arsR operon evolution

(A) Schematic outline of arsR operon evolution. (B) Improvement of biosensor induction activity towards arsenite after three rounds of evolution (The calculation methods are described in the Supporting Information). WT refers to the wild-type arsR operon. Ep1, ep2 and ep3 are the first, second and third round evolution mutants. The bacteria were induced with arsenite at the concentration of 75 μg/L for 1 h at 37 °C. The first round evolution mutants exhibited up to 7-fold improvement in response to arsenite. The second and third round evolution mutants continued this trend, and the final mutant ep3 exhibited up to 12-fold increase in induced response. Standard deviations are marked with error bars (n=8).

1. High sensitive arsenite biosensor mutants obtained through directed evolution.

To improve the sensitivity of arsenite-responsive biosensor, we constructed an arsenite-inducible vector with *GFP* as reporter gene, pUC18-ars-gfp. Then, iteratively multi-rounds of directed evolution were applied to improve the arsenite-sensing element and seek for the improved mutants. For each round of directed evolution, we first created a random gene mutation library of arsenite-inducible promoter through ep-PCR in *E.coli* strain DH5α, which contained over 1,000,000 variants. Since the evolution of an arsenite-inducible biosensor aimed for both low genetic background and optimal gene expression levels, the library was subjected to flow cytometric sorting with a bi-directional screening scheme (Figure 1A). Mutants from each library were characterized through sequence analysis and arsenite induction test. The mutants with the best performance were picked up as the ep-PCR template for next round of library construction.

As shown in Figure 1B, the mutants from each generation resulted in an increased induction of GFP fluorescence intensity. Induction response of a final mutant ep3 was increased more than 12 fold compared with that of the wild-type control (Figure 1B).

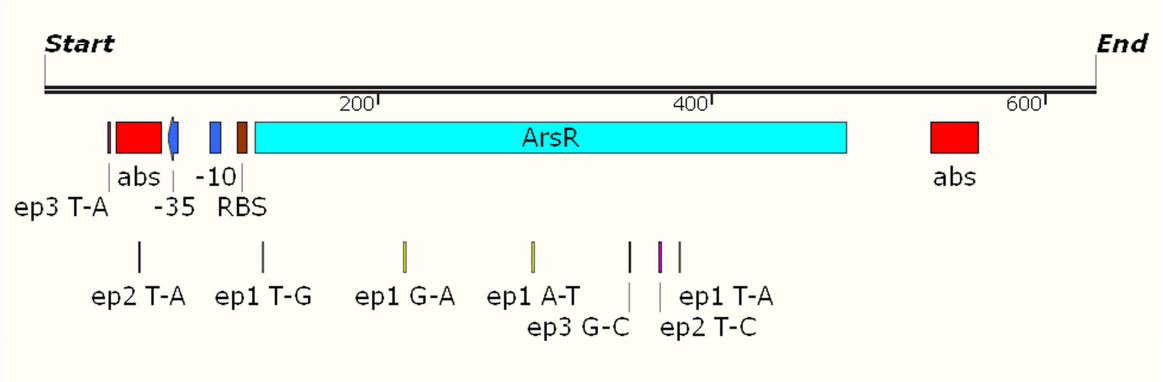

Figure 2. A schematic mutation site summary of the arsR mutants. Ep1, ep2 and ep3 are the first, second and third round evolution mutants.

2. Sequence analysis of evolved arsenite biosensor mutants

The sequence of the wild-type gene operon and three variants from each round of evolution were aligned and the relevant mutation site are marked (Figure 2 and Figure S3, Supporting Information).

In arsenite-responsive biosensors, arsenite is detected by ArsR repressor protein, which has the sequence Cys32-Val-Cys34-Asp-Leu-Cys37 in the DNA binding domain.[13] The C32 and C34 cysteine residues form a very specific binding site for arsenite.[27] By sequence analysis of three evolved mutants from iterative round of screening, the mutation took place in the arsR regulatory protein outside the two cysteine residues, and thus would not affect the specificity to arsenite. The mutation sites on the arsR repressor and on the repressor binding site might be involved in the dissociation of the repressor from its binding site and improvement of the affinity of the repressor with DNA binding site. The first generation of evolved mutant ep1, exhibited a lower genetic background with approximately 2-fold decrease in basal arsR expression (see upper left

figure in Figure 3). All four point mutations of ep1 in the nucleotide sequence located in arsR repressor, two of which further resulted in two amino acids substitutions (Leu2Trp and Glu56Val). The second round of evolution mutant ep2 had a point mutation at the 243th site of arsR gene, and an additional mutation site located on the 14th position of its upstream ArsR DNA binding site (abs). Compared to the mutation sites of ep1, the mutation at the arsR binding site mihgt be the key reason that led to the improvement of mutant ep2 in producing a higher fluorescence response to arsenite. The ep3 mutant from the final round of evolution had two point mutations located on the 225th site of arsR protein. The final mutant ep3 extended the evolutional trends of ep1 and ep2 with stable improvement of induction response to arsenite. The mutation sites found during the three rounds of evolution might give some implications on the sensing mechanism of arsenite-sensing biosensor. For instance, site-directed mutagenesis of the nucleotide found in ep3 mutant one by one could help to understand the role of each nucleotide in improving the sensitivity of arsenite-responsive biosensors. Further DNA and protein interaction will be needed for in-depth interpretation of the exact mechanism that causes the improvement of biosensor.

3. Analytical performance of the evolved biosensor to arsenite

In this study, the biosensor plasmid was transformed into *E.coli* DH5α. In *E.coli* strain, there existes arsenate reductase enzyme, which could convert intracellular arsenate to arsenite.[28] So the biosensor could response to both arsenite and arsenate (Figure S4, Supporting Information). Since arsenite is around 60 times more toxic than arsenate, we will focus mainly on the detection of arsenite in this study.[29]

Since the mechanism of arsenite-responsive bacterial biosensor is based on its natural defense system against arsenite and the whole biosensor is working as a living system, continuous incubation with arsenite will lead to increased gene expression and GFP accumulation. The performance of arsenite bacterial biosensor is not only related to the arsenite concentration, but also dependent on incubation time and the cellular growth state. The performance of the final evolved mutant ep3 was examined in comparison with that of the wild-type biosensor.

3.1 Dose-dependent response to arsenite

To examine the dose response of ep3 mutant to arsenite, the wild-type arsenite biosensor and the evolved arsenite biosensor were induced respectively, by a series of designated concentrations of arsenite for 1 h at 37 °C. Both of them displayed a dose-dependent induction response (Figure 3) . A good linear response of the biosensor to arsenite occurred between 0 and 75 μg/L. Obviously, the fluorescence of ep3 biosensor increased more significantly than that of the wild-type after arsenite induction. The upper left panel of Figure 3 showed that the detection limit of As (III) had improved from 3 μg/L to 0.75 μg/L (black square-ep3; open square-wild-type). Moreover, the background GFP emission of the evolved biosensor was declined more than 2-fold (Fluorescence from 1200 to 500). These results indicate that the evolved ep3 biosensor is more sensitive than the wild-type one.

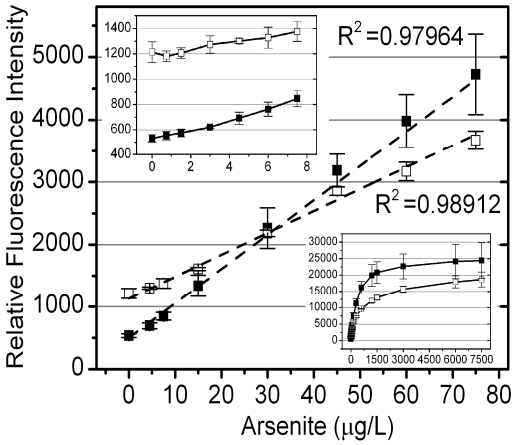

Figure 3. Dose-dependent arsenite-induced GFP fluorescence of the *E. coli* (pUC18-ars-gfp, open squares) and *E. coli* (pUC18-ep3ars-gfp, black squares) strains. Average fluorescence of each strain was achieved after incubation with different arsenite concentrations for 1 h at 37°C. Error bars indicate the calculated average deviation from the average fluorescence value for the whole measured population of cells (total of 10000 cells) with the standard deviation of three replicates.

3.2 Time-dependent response to arsenite

As biosensor for arsenite detection in practical application, the response time is an important factor that should also be considered. Two typical arsenite concentration of both 10 μg/L (European countries and the United States arsenic permission level) and 50 μg/L (China and other countries arsenic permission level) were chosen to perform this assay. In this time response assay, the bacteria were exposed to arsenite for different incubation time intervals ranging from 15 to 135 minutes. A time-dependent arsenite detection assay with both the wild-type and ep3 biosensor was shown in Figure 4. Under the concentration of 10 μg/L As(III), the evolved ep3 arsenite biosensor exhibited more than a 2-fold fluorescence increase within 45 minutes, while

the wild-type biosensor took over 90 minutes to reach that amount of fluorescence increment. The results indicate that the response of evolved ep3 biosensor to arsenite is earlier than that of the wild-type one.

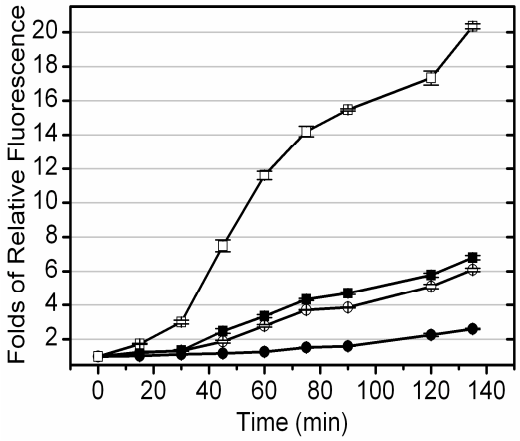

Figure 4. Arsenite measurements with the *E. coli* (pUC18-ars-gfp, circle) and *E. coli* (pUC18-ep3ars-gfp, squares) strains with different induction time at two arsenite concentrations, 10 (black symbol) and 50 (open symbol) μg/L. Average fluorescence of each strain was achieved after incubation with different arsenite concentrations in a serials of incubation time span from 15 to 135 minutes at 37°C. Error bars indicate the calculated average deviation from the average fluorescence value for the whole measured population of cells (total of 10000 cells) with the standard deviation of three replicates.

3.3 Specificity of the evolved ep3 biosensor

Since improved performance of arsenite-responsive biosensor was achieved through construction of random mutagenesis libraries and flow cytometric sorting, the evolved biosensor might lose its specificity due to the random mutations in the key regulation component. In order to test the

specificity of the evolved ep3 biosensor, the response of the evolved ep3 biosensor to some of the most common chemicals that are of major public health concern were tested (Figure 5). Arsenite and antimony were prepared in the concentration of 20 μg/L, while all the other chemical compounds were prepared in 5 mg/L solution (Refer to Annotation V in the Supporting Information). As shown in Figure 5, the evolved ep3 arsenite-responsive biosensor had high affinity to arsenite, but no visible affinity to other chemicals, indicating that the specificity did not change through the *in vivo* evolution.

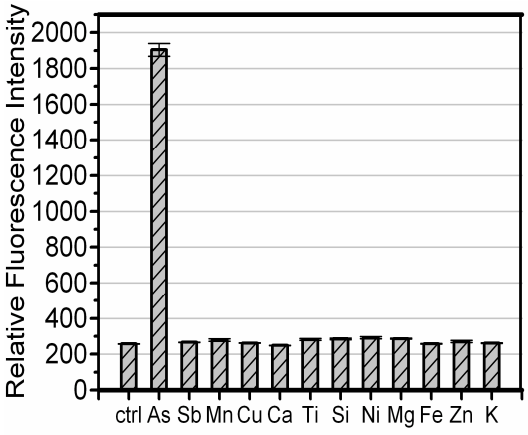

Figure 5. Specificity test of arsenite-responsive bacterial biosensors. Some of the most common chemicals that are of major public health concern were examined. Arsenite and antimony were prepared in the concentration of 20 μg/L, and all the other compounds were prepared in 5 mg/L solution. Average fluorescence of each strain was achieved after induction with different element for 1 h at 37°C. Error bars represent standard deviation from the mean (n=3).

3.4 Effect of bacterial growing on arsenite detection

For the needs of practical applications, the convenient operation is another factor that should be considered. The conventional induction assay with biosensors was usually performed with exponentially growing bacteria (about 0.4 OD600). By comparing the arsenite-induced

fluorescent signals, Figure 6 showed that the bacteria in stationary phase was more sensitive than that in log phase, and the diluted overnight culture could directly be used for induction check with better fluorescence performance, especially with the evolved ep3 biosensor. The induction assay with the diluted stationary phase cell skipped the preparation of log phase cell, which simplified experimental operation and saved time.

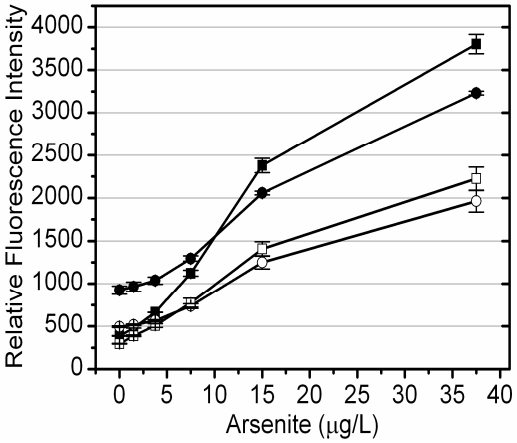

Figure 6. Arsenite measurements with the *E. coli* (pUC18-ars-gfp, circle) and *E. coli* (pUC18-ep3ars-gfp, squares) strains with different induction scheme. Stationary phase cells (black symbol) were induced directly with arsenite from diluted overnight cell culture with no further incubation. While log phase cell (open symbol) were induced with arsenite after pre-incubating diluted overnight cell culture to log phase (OD600=0.4). Average fluorescence of each strain was achieved after induction with different arsenite concentrations for 1 h at 37°C.Error bars indicate the calculated average deviation from the average fluorescence value for the whole measured population of cells (total of 10,000 cells) with the standard deviation of three replicates.

4. Effect of natural water on ep3 arsenite biosensor.

Since the bacterial biosensor functions as a living organism, and the detection is performed by mixing biosensor cell culture with water sample of interest in a 1:1 ratio. The complex nature water components might affect the growth and the well-being of bacterial biosensor, and in some cases leading to false arsenite detection response. Moreover, the complex environmental factors in nature water might affect the occurrence state of target substrate that caused it inaccessible to the biosensor.[30] Therefore, we prepared a serial of artificial arsenite contaminated nature water with deionized water, tap water, well water and reservoir water to test the performance of evolved ep3 bacterial biosensor. As shown in Figure 7, the evolved arsenite-responsive biosensor ep3 performed steady in all four artificial arsenite contaminated water sample, and thus could work as a routine analytics. for monitoring arsenite.

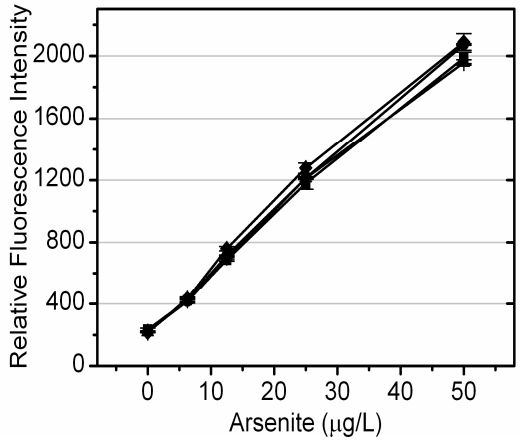

Figure 7. Effect of nature water on ep3 arsenite biosensor. Four different water sample of deionized water (square), tap water (triangle), well water (circle) and reservoir water (diamond) were subjected to a serial of standard arsenite solution preparation(0, 6, 12.5, 25, and 50 μg/L As(III)), in order to test interference of iron or other complex compounds in environmental water sample. Average fluorescence of cells was achieved through incubation with increase of arsenite concentrations at 37°C.

340

341 5. Arsenite detection assay with ep3 baterial biosensor in arsenite contaminated groundwater.

342 ICP-MS and the evolved arsenite-responsive biosensor assay were used in parallel to measure
343 arsenite concentrations in groundwater sample collected in September 2014 from Togtoh regions,
344 Inner Mongolia. To give an estimated arsenite concentration value, standardized incubation with
345 known arsenite concentrations were carried out under the same conditions in comparison with
346 the arsenite contaminated water sample. Arsenite calibration curves were prepared from
347 commercially available arsenite stock solution of 1000 mg/L and established with final induction
348 concentrations of 0, 1, 2, 5, 10, 25 and 50 μg/L, respectively. A good linear response of ep3
349 biosensor to arsenite occured between 0 to 50 μg/L (Figure 8). In order to eliminate the negative
350 influence of complicated compositions in environmental water on the response of the biosensor
351 to arsenite, the Inner Mogolia groundwater sample was subjected to a serials of dilution rate of
352 1:4, 1:10, 1:20, 1:40 and 1:100 before the induction assay. ICP-MS detection with the diluted
353 groundwater samples were used to verify the biosensor test. The data from Table 2 showed that
354 the average value of arsenite in groundwater sample estimated by ICP-MS was 84.831 μg/L,
355 while that estimated by the ep3 biosensor is 74.024 μg/L. This result indicates that the evolved
356 ep3 biosensor functions well in prediction of arsenite concentration in natural water sample.

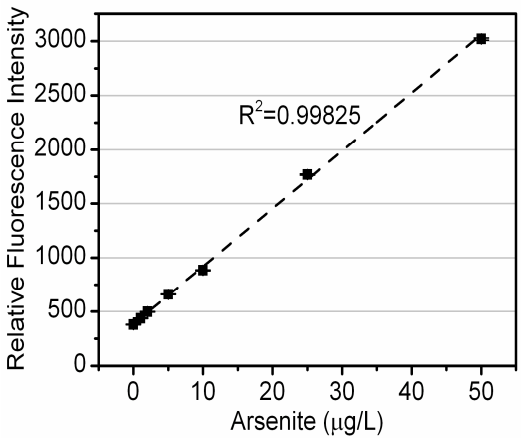

Figure 8. Calibration of arsenite with concentration range from 1 to 50 μg/L. Slope linearly interpolated from averages from triplicate incubations of arsenite for one hour at 37℃. Concentration calculated from hyperbolic slope (y=383.930+53.530x; R^2=0.998).

Table 2. ICP-MS and ep3 arsenite-responsive biosensor detection of arsenite contaminated groundwater. (* mean were calculated with all the dilution sample values.)

Water Sample	Relative Fluorescence Intensity (Mean)	ICP-MS (μg/L)	Estimated ICP-MS (μg/L)	Calculated according to trend-line (μg/L)	Estimated Concentration(μg/L)
original	N.A	85.131	85.131	-	-
1:4 dilution	1305.000	20.819	83.276	17.207	68.828
1:10 dilution	821.333	8.960	89.600	8.171	81.710
1:20 dilution	567.667	4.548	90.960	3.432	68.640
1:40dilution	485.667	2.178	87.120	1.901	76.040
1:100dilution	424.000	0.732	73.200	0.749	74.900
*Mean			84.831		74.024

This work was the first effort of the artificial evolution of an arsenite inducible biosensor with high-throughput screening. The results suggest that manipulating the key genes in the biosensor could effectively improve the performance of target biosensor. The method in this study provides a route to optimize performance of substrate inducible biosensor through the creation of random mutagenesis libraries and bi-directional FACS strategies. The results clearly demonstrate that the evolution of substrate inducible biosensor could effectively improve sensitivity and the detection limit, which might have a profound impact on further practical application of the biosensor.

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We are grateful to Dr J.R. van der Meer (University of Lausanne, Switzerland) for his generous gift of pPR-arsR-ABS plasmid. We would also thank Dr. Xingjiu Huang (Chinese Academy of Sciences, China) for providing Inner Mongolia groundwater.

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Supporting Information

Table S1-S2 and Figure S5 showed the specifity test of evolved EP3 biosensor with three different concentrations. Figure S1 showed the schematics of the construction of plasmid of the wild-type arsenite-responsive biosensor (pUC18-ars-gfp) and a null variant (pUC18-AID-gfp). In Figure S2, an example was set up to illustrate how the performance of arsenite-responsive

biosensor was evaluated. Sequence alignment of the wild-type *arsR* gene and three variants was shown in Figure S3 with every mutation site highlighted in grey. The response of evolved ep3 biosensor to arsenate was monitored and the result was shown in Figure S4. This information is available free of charge via the Internet at http://pubs.acs.org.

Abbreviations

As(III), arsenite; abs, ArsR DNA binding site; ep-PCR, error-prone PCR.

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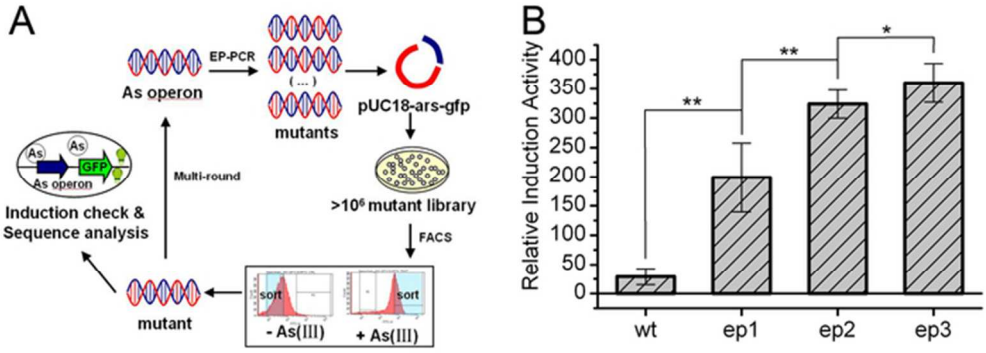

Figure 1. arsR operon evolution
(A) Schematic outline of arsR operon evolution. (B) Improvement of biosensor induction activity towards arsenite after three rounds of evolution (The calculation methods are described in the Supporting Information). WT refers to the wild-type arsR operon. Ep1, ep2 and ep3 are the first, second and third round evolution mutants. The bacteria were induced with arsenite at the concentration of 75 μg/L for 1 h at 37 °C. The first round evolution mutants exhibited up to 7-fold improvement in response to arsenite. The second and third round evolution mutants continued this trend, and the final mutant ep3 exhibited up to 12-fold increase in induced response. Standard deviations are marked with error bars (n=8).

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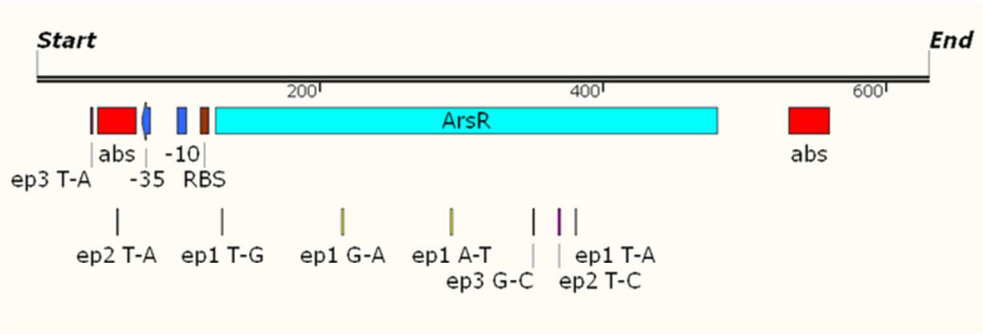

Figure 2. A schematic mutation site summary of the arsR mutants. Ep1, ep2 and ep3 are the first, second and third round evolution mutants.
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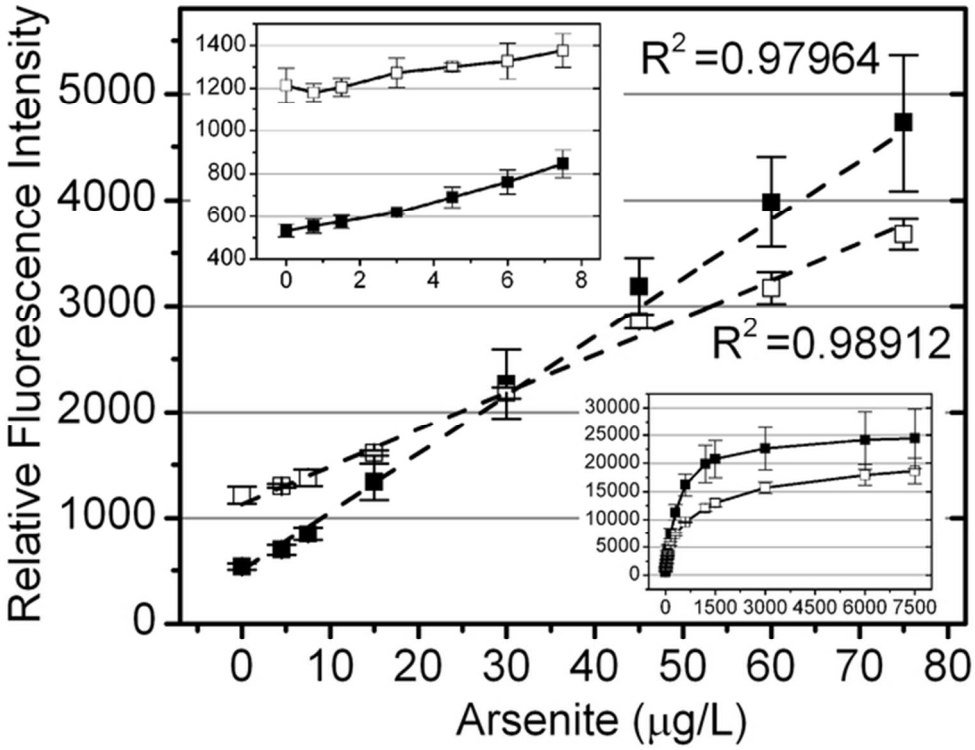

Figure 3. Dose-dependent arsenite-induced GFP fluorescence of the E. coli (pUC18-ars-gfp, open squares) and E. coli (pUC18-ep3ars-gfp, black squares) strains. Average fluorescence of each strain was achieved after incubation with different arsenite concentrations for 1 h at 37°C. Error bars indicate the calculated average deviation from the average fluorescence value for the whole measured population of cells (total of 10000 cells) with the standard deviation of three replicates.
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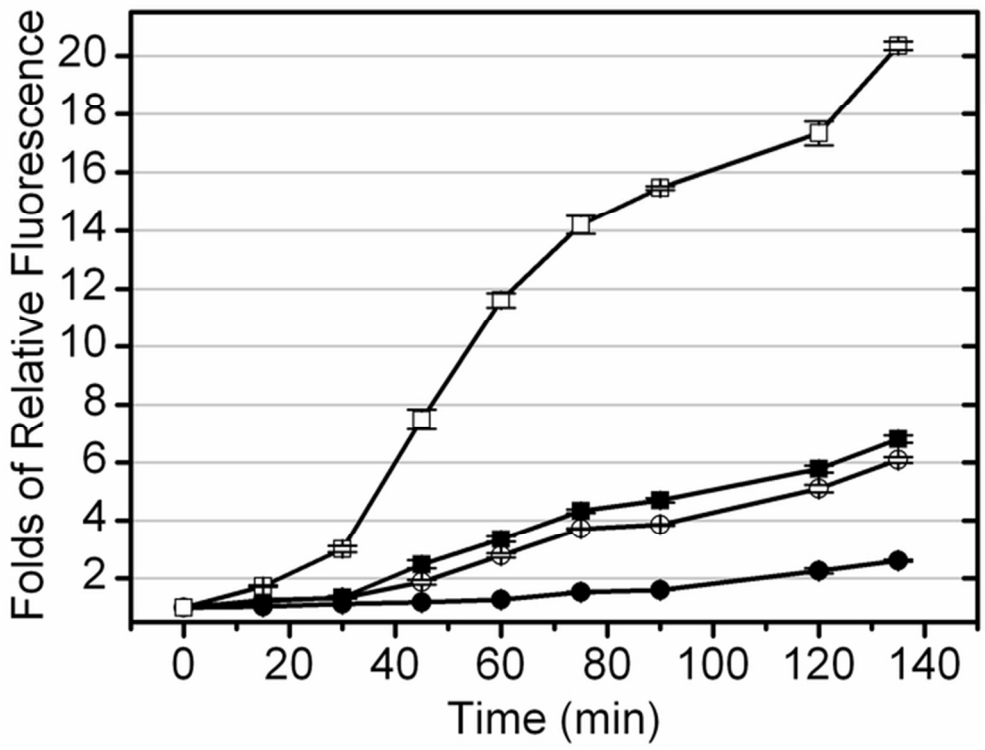

Figure 4. Arsenite measurements with the E. coli (pUC18-ars-gfp, circle) and E. coli (pUC18-ep3ars-gfp, squares) strains with different induction time at two arsenite concentrations, 10 (black symbol) and 50 (open symbol) μg/L. Average fluorescence of each strain was achieved after incubation with different arsenite concentrations in a serials of incubation time span from 15 to 135 minutes at 37°C. Error bars indicate the calculated average deviation from the average fluorescence value for the whole measured population of cells (total of 10000 cells) with the standard deviation of three replicates.
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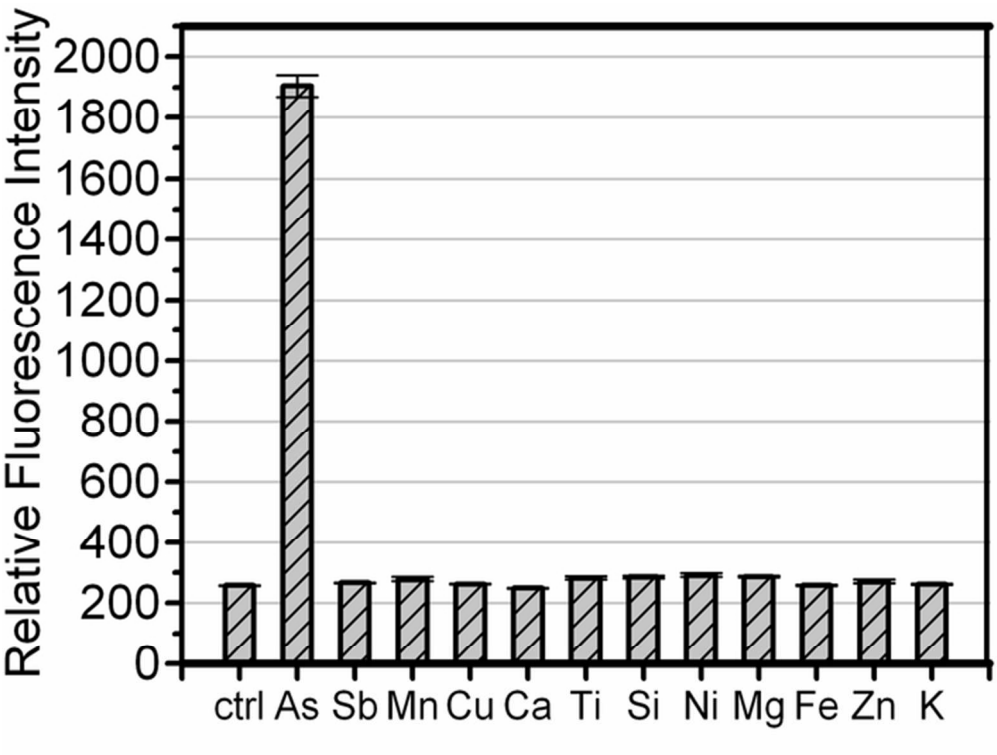

Figure 5. Specificity test of arsenite-responsive bacterial biosensors. Some of the most common chemicals that are of major public health concern were examined. Arsenite and antimony were prepared in the concentration of 20 μg/L, and all the other compounds were prepared in 5 mg/L solution. Average fluorescence of each strain was achieved after induction with different element for 1 h at 37°C. Error bars represent standard deviation from the mean (n=3).
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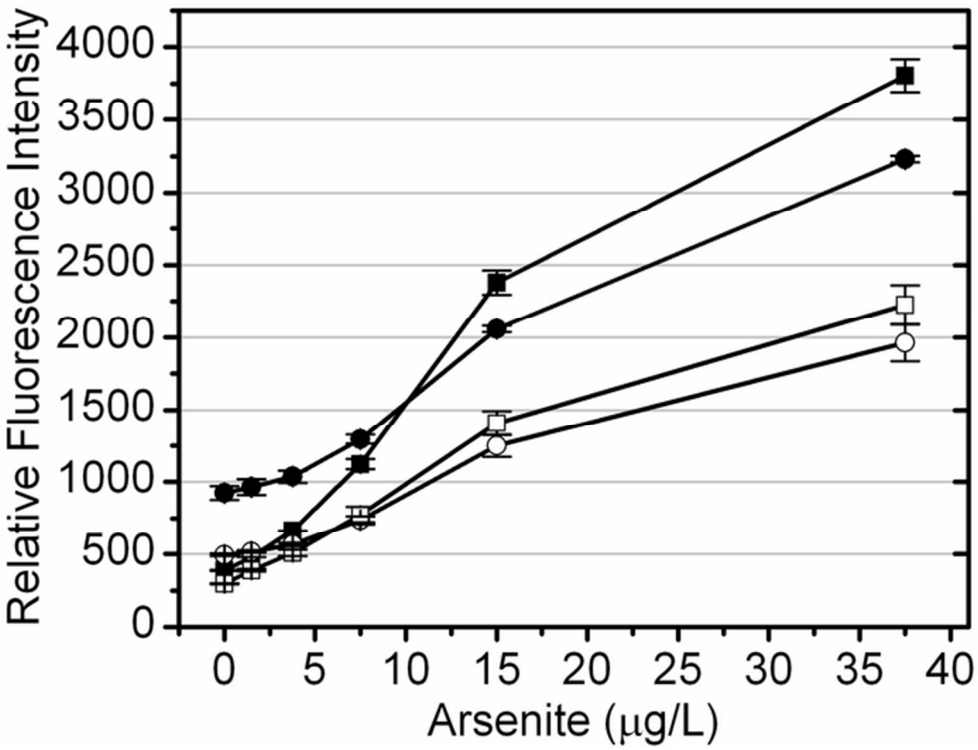

Figure 6. Arsenite measurements with the E. coli (pUC18-ars-gfp, circle) and E. coli (pUC18-ep3ars-gfp, squares) strains with different induction scheme. Stationary phase cells (black symbol) were induced directly with arsenite from diluted overnight cell culture with no further incubation. While log phase cell (open symbol) were induced with arsenite after pre-incubating diluted overnight cell culture to log phase (OD600=0.4). Average fluorescence of each strain was achieved after induction with different arsenite concentrations for 1 h at 37°C.Error bars indicate the calculated average deviation from the average fluorescence value for the whole measured population of cells (total of 10,000 cells) with the standard deviation of three replicates.
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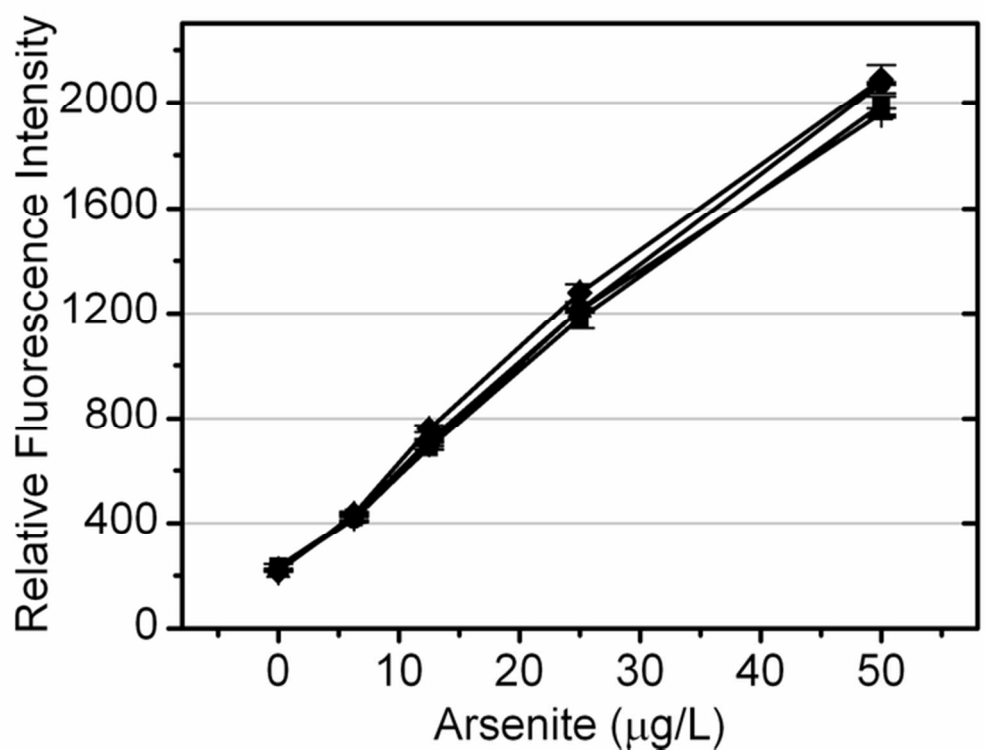

Figure 7. Effect of nature water on ep3 arsenite biosensor. Four different water sample of deionized water (square), tap water (triangle), well water (circle) and reservoir water (diamond) were subjected to a serial of standard arsenite solution preparation(0, 6, 12.5, 25, and 50 μg/L As(III)), in order to test interference of iron or other complex compounds in environmental water sample. Average fluorescence of cells was achieved through incubation with increase of arsenite concentrations at 37°C.
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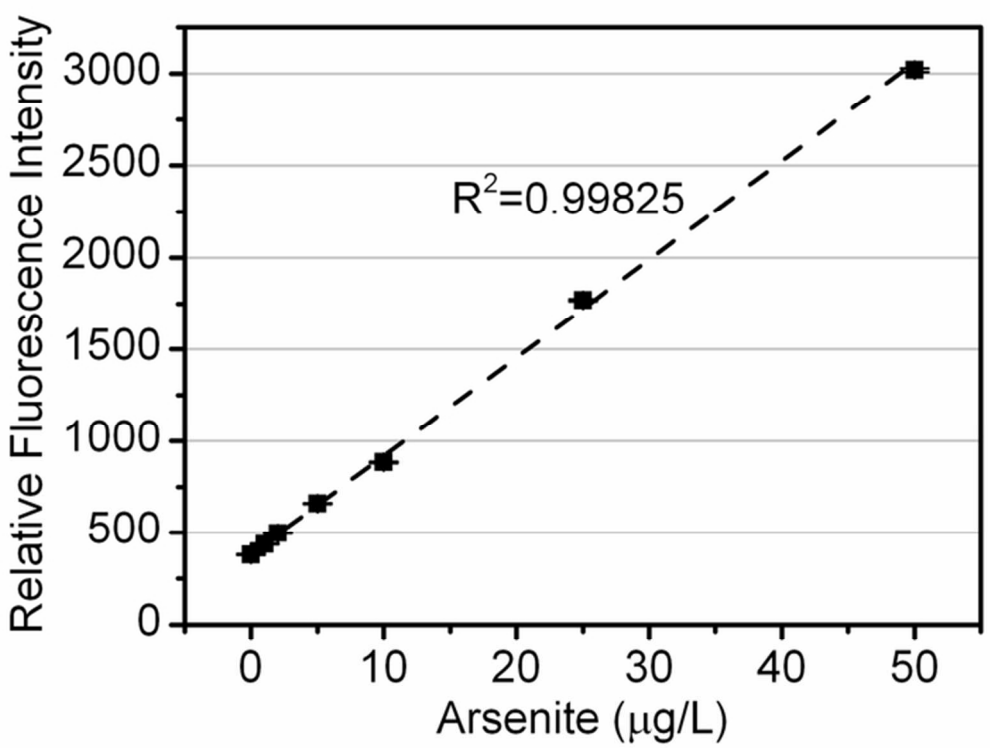

Figure 8. Calibration of arsenite with concentration range from 1 to 50 μg/L. Slope linearly interpolated from averages from triplicate incubations of arsenite for one hour at 37°C. Concentration calculated from hyperbolic slope (y=383.930+53.530x; R2=0.998).
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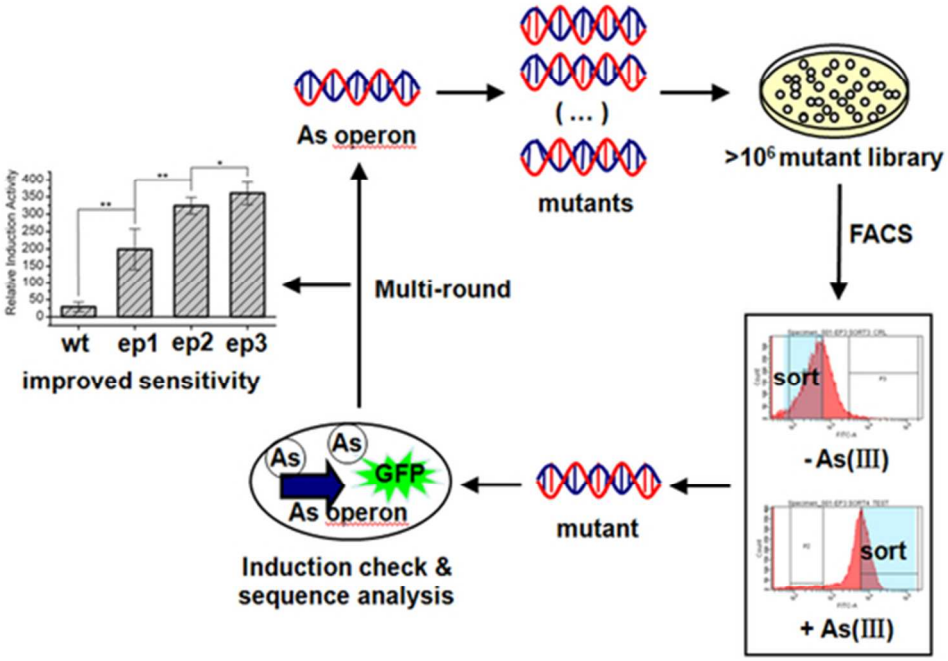

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