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Article

Evolved Biosensor with High Sensitivity and Specificity for Measuring Cadmium in Actual Environmental Samples

Yeshen Cai, Kaili Zhu, Liang Shen, Jie Ma, Lingzhi Bao, Dongdong Chen, Liangchen Wei, Nan Wei, Binmei Liu, Yuejin Wu, and Shaopeng Chen*

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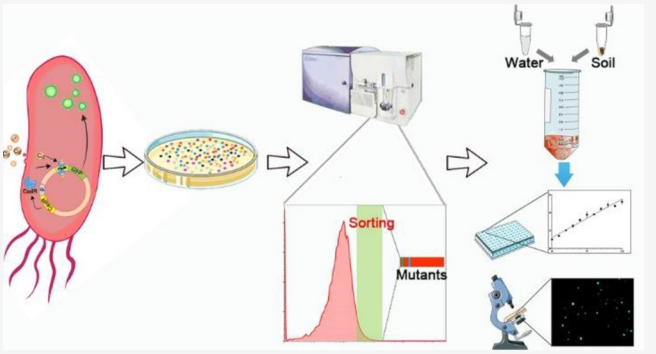
ABSTRACT: Bacterial biosensors have great potential in contaminant detection for sensitivity, specificity, cost-effectiveness, and easy operation. However, the existing cadmium-responsive bacterial biosensors cannot meet the real-world detection requirements due to lack of sensitivity, specificity, and anti-interference capability. This study aimed to develop a bacterial biosensor for detecting the total and extractable cadmium in actual environmental samples. We constructed the cadmium-responsive biosensor with the regulatory element (cadmium resistance transcriptional regulatory, CadR) and the reporting element (GFP) and improved its performance by directed evolution. The mutant libraries of biosensors were generated by error-prone PCR and screened by continuous five-round fluorescence-activated cell sorting (FACS), and a bacteria variant epCadR5 with higher performance was finally isolated. Biosensor fluorescence intensity was measured by a microplate reader, and results showed that the evolved cadmium-responsive bacterial biosensor was of high sensitivity and specificity in detecting trace cadmium, with a detection limit of 0.45 μg/L, which is 6.8 times more specific to cadmium than that of the wild-type. Furthermore, microscopic qualitative analysis results showed that the bacteria could produce fluorescence response in a cadmium-contaminated soil matrix, and quantitative analysis results showed that the values of cadmium from epCadR5 bacteria were close to that from inductively coupled plasma-mass spectrometry. These results suggest that the biosensor may have a broad application prospect in the detection of cadmium-contaminated soil and water.



KEYWORDS: *Cadmium, bacterial biosensor, directed evolution, FACS, actual sample detection*

INTRODUCTION

Cadmium (Cd) is one of the primary environmental pollutants existing in a small amount in nature, mostly in the form of cadmium sulfide and a small amount in zinc ore.[1] The environmental release of cadmium mainly comes from industrial production, including metal mining, cadmium-containing goods production, phosphate fertilizer abuse in agricultural production, and the combustion of fossil fuels.[2] Cadmium is not an essential element for the human body. The presence of trace amounts can pose a significant threat to human health. The source of human absorption is mainly food and water.[3] The long-term consumption of cadmium-containing food and drinking water can lead to diseases like abdominal pain and fragile bones, as well as even a variety of cancers.[4] Consequentially, cadmium is listed as a highly hazardous toxic substance and carcinogen by the European Union. The World Health Organization (WHO) has reduced the limit of cadmium in drinking water from 5 to 3 μg/L.[5] Thus, the detection of cadmium in the environment is of great significance to prevent the health risk from cadmium.

The traditional methods for the detection of cadmium mainly rely on highly sensitive chemical analytics, such as atomic fluorescence spectrometry (AFS), atomic absorption spectrophotometry (AAS), and inductively coupled plasma-mass spectrometry (ICP-MS). However, these laboratory-based assays for cadmium quantification are uneconomical, time consuming, and complex, in which pretreatment is usually essential, especially in complex sample analyses. In the process of pretreatment, cadmium in the chelated form will be converted to an ionic state with the use of a strong acid, which is unfriendly to the environment and cannot be applied in on-site detection for assessing the actual bioavailability.[6] The whole-cell biosensors constructed by genetic engineering sensors and bacteria as carriers can effectively solve these

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problems. Cadmium whole-cell biosensors are constructed with the CadR/CadC gene as the sensor element and the reporter gene as the signal generator.[7] To improve the performance of cadmium biosensors, synthetic biotechnologies and designs have been adopted. By Gibson assembly technology with *Pseudomonas aeruginosa* promoter (pCadR), the micromolar concentrations of cadmium can be measured.[8] Using different combinations of detection elements, Jia et al. designed 30 whole-cell cadmium biosensors and picked a biosensor with the best performance. Combined with a positive feedback amplifier, the detection limit of the sensor achieved 1.12 μg/L in water samples.[9] As the core element, cadmium-responsive transcription activators, CadR or CadC, bind with cadmium and regulate the expression of the reporter gene. This function of CadR/CadC is vital to determining the performance of cadmium biosensors.[10] However, due to lower sensitivity and specificity, the biosensors with wild-type CadR/C cannot meet the actual detection requirements, especially in complex environmental scenarios, such as oil, wastewater, and heavy metal-rich tannery sludge. Complex environmental factors, such as Pb^{2+} and Zn^{2+}, can combine with wild-type CadR and compete with Cd^{2+}, leading to the assay results deviating from the actual values.

Other approaches in molecular and synthetic biologies have successfully improved the sensitivity of biosensors, such as modulating promoters,[11,12] reporter genes,[13] and SD sequence.[13] However, these approaches do not refer to the transcription regulatory elements, so they cannot be applied in increasing the specificity of biosensors. Directed evolution is a useful tool for a performance boost of proteins through continuous mutation and screening, such as antibodies,[14] fluorescent proteins,[15] and enzymes.[16] The directed evolution improves the performance of biosensors and has been applied in recent years. The directed evolution of transcriptional regulatory protein can improve the sensitivity, specificity, and other performances of biosensors.[17,18] Hence, directed evolution was employed in this study to generate a cadmium-responsive bacteria with high performance.

The biosensors reported in recent years for the detection of environmental contaminants are limited to the detection of water samples,[19−21] even those obtained by manual spiking.[22−24] In addition, there is a lack of evaluation of the actual environmental application ability of the biosensors, especially during on-site detection. For the qualitative and quantitative detections of trace cadmium in a complex environment, in this study, we tried to isolate a cadmium-responsive bacteria with high anti-interference capability, which could meet the real-world detection requirements.

For this purpose, we constructed an error-prone mutant library of CadR based on a wild-type cadmium-responsive bacteria, and the mutants with stronger specificity and higher sensitivity were screened by the flow bidirectional fluorescence-activated cell sorting system. After five rounds of directed evolution in vivo, we obtained a mutant epCadR5 with a detection limit and specificity of 0.45 μg/L and 1.97, which were 2.6 and 6.8 times higher than that of the wild-type CadR in water samples, respectively. The detection results of actual environmental samples showed that the evolved bacteria were comparable with ICP-MS in relatively simple environmental samples, and it could even produce stable fluorescence expression in complex environmental soils. This study has provided a promising and feasible approach for direct cadmium detection in an actual environment using whole-cell biosensors.

MATERIALS AND METHODS

Strains, Cultivation of Bacteria, and Chemical Components. Bacterial strains, chemicals, and culture media used in this study are shown in the Supporting Information Text S1. All bacteria were cultured in a shaking incubator at 37 °C at 200 rpm.

Construction of Wild-Type Cadmium-Induced Biosensor. A wild-type cadmium-induced whole-cell biosensor plasmid was first constructed by recombining a cadmium resistance gene (CadR) and promoter gene (pCadR). CadR and pCadR were PCR amplified with corresponding primers (Table S1), and the products were digested by restriction endonuclease and inserted into vector pSB1k3-mAID-Kana-GFP to construct expression plasmid *E. coli* JudeI/pSB1k3-CadR-Kana-GFP. The expression of a green fluorescent protein gene is regulated by cadmium. Cadmium, as a switch for regulating the expression of GFP, can activate the bacteria to produce different intensity fluorescence responses to different concentrations of cadmium.

Bacteria Activation. Bacterial clones were cultured overnight in 3 mL of LB-Amp medium. The overnight culture solution was diluted with 10% 3-(N-morpholino) propanesulfonic acid (MOPS) medium and then cultured to the logarithmic growth stage (OD_{600} = 0.4−0.6). A cadmium standard solution or an actual sample solution was added to the induction system to the final concentration as the preset value and incubated at 37 °C for 2 h. After centrifugation, the induced bacterial liquid was washed with deionized water and then made into a bacterial suspension for fluorescence detection.

Directed Evolution of CadR. *Construction of CadR Mutant Library.* PCR mutagenesis of the protein coding region in the CadR was carried out by error-prone PCR (ep-PCR). The gene fragments containing 543 base pairs encoding cadmium-sensitive elements and a constitutive promoter were amplified with error-prone primers F-epCadR and R-epCadR (Table S2). Taq DNA polymerase was used, and a CadR mutant library with an approximately 3% mutation rate was obtained by adding extra nucleotides and Mn^{2+} to regulate the mutation rate.[25] Ep-PCR products were ligated into ApaI/*Bam*HI-digested pSB1K3-mAID-Kana-GFP, and then, recombinant plasmids were electrotransformed into *E. coli.* JudeI competent cells with a MicroPulse electroporator (BioRad).[26] The bacterial solutions after electroporation were incubated for 1 h at 37 °C, then coated evenly on 150 mm LB solid medium containing 2‰ kanamycin overnight at 37 °C. All clones were scraped off the Petri dish the next day with a coating stick. All the colonies were scraped and collected, and the plasmid of the mutant library was extracted with a plasmid maxiprep kit (Biomiga). The mutant library contains approximately 10,000,000 clones.

Sorting and Identification of Mutant Library. The mutant library plasmid was re-electrotransformed into *E. coil* JudeI competent cells, and bacterial suspensions with and without cadmium induction were prepared as described above. The cadmium-induced bacteria have a low background without inducer but produce a high fluorescence response quickly when cadmium is present. The library's mutants with a higher fluorescence response were sorted by fluorescence-activated cell sorting (FACS) as described in Text S1 of the Supporting Information. Overall, five rounds of continuous evolution were carried out, and the final mutant epCadR5 was obtained.

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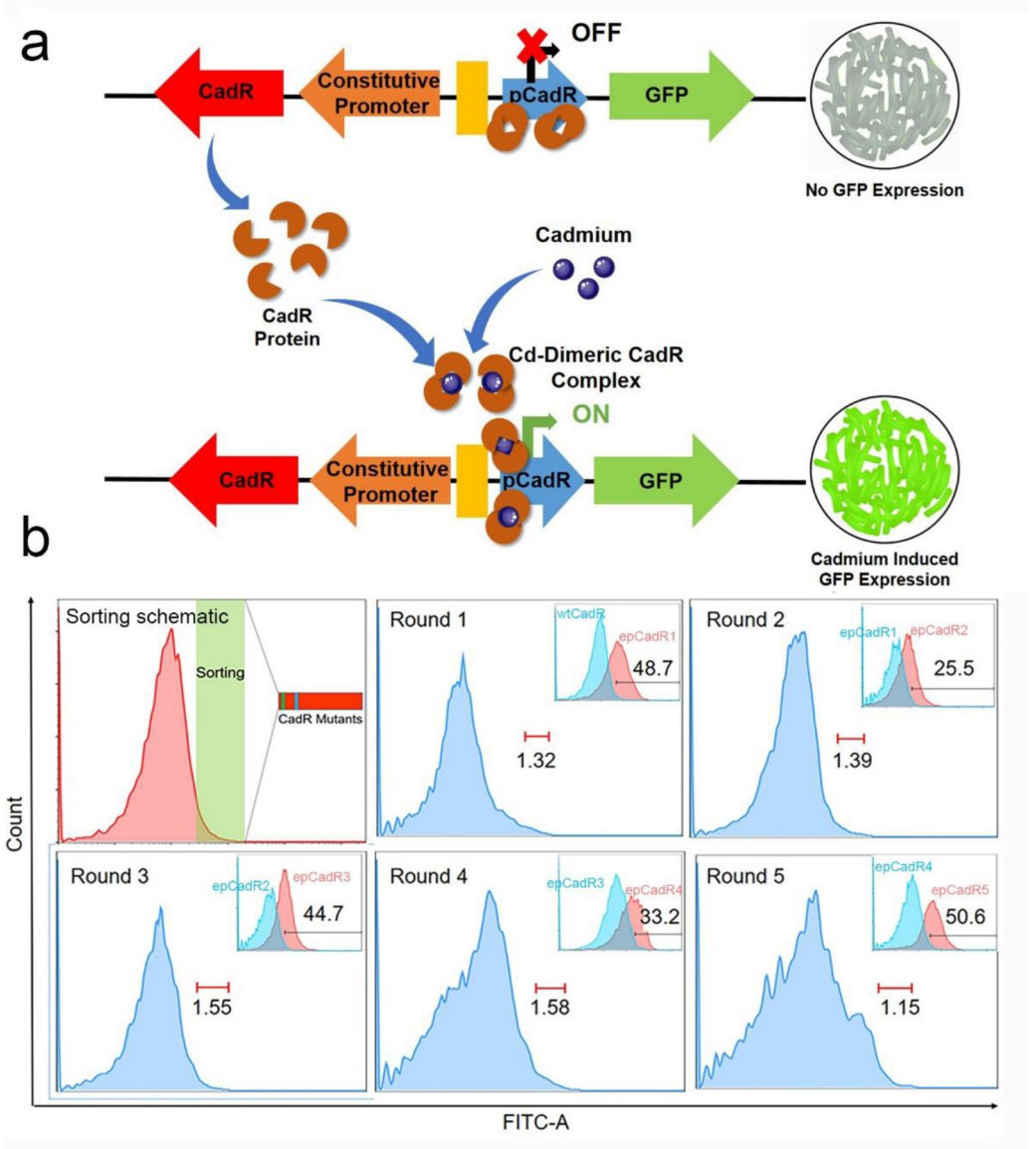

Figure 1. (a) Strategies for construction of wild-type cadmium-induced bacteria. (b) Directed evolution and screening of CadR mutants. A total of five rounds of screening were carried out, and the cadmium concentrations in each round were 500, 200, 100, 10, and 5 μg/L, respectively. The red line marker represented the selected positive mutants.

Sequence alignment of five rounds of evolved mutants was confirmed by sequence alignment programs (ClustalW).

Performance Detection and Fluorescence Measurement of Mutants. The dose-response, time-response, and cadmium-specific response of the bacteria before and after evolution were verified to analyze the performance of the bacteria in practical detection applications. Measurements of the OD_{600} and fluorescence value were carried out using a microplate reader (SpectraMax i3). The relative fluorescence intensity (RFI) was used to express the power of the fluorescent signal produced by bacteria in response to cadmium. The value of RFI was calculated as (sample fluorescence value/OD_{600}) divided by (background fluorescence value/background OD_{600}).[9]

Real Environmental Sample Detection with Evolved Bacterial Biosensor. *Soil Sampling and Preparation.* A total of seven soil and water samples (0−20 cm) were collected from rivers and farmland near a pyrite mining area in Tongling City, Anhui Province, China (Figure S1). Sampling was carried out in August, and sampling sites were distributed along rivers, tributaries, and reservoirs within 10 km of the mining area. A cadmium-contaminated experimental field was simulated by applying $CdCl_2{\cdot}5H_2O$ to obtain artificial contamination of cadmium soil, and $CdCl_2{\cdot}5H_2O$ in deionized water was poured into the cadmium-free farmland soil as it was well mixed to a final concentration of 19.8 mg/kg.[27] The soils of the experimental field and the control field (cadmium-free farmland soil) were collected using the same method. Before analysis, the soil samples were dried at room temperature and homogenized with a 100-mesh nylon screen. Water samples were filtered and sterilized with a 0.22 μm Millipore filter. Air-dried soil samples (0.2 ± 0.01 g) were soaked, extracted using 25 mL Milli-Q water, and processed by ultrasound for 1 h. Series dilutions of the water extraction were performed to ensure that the total cadmium content was within the detection range of the bacteria.

Measurement of Cadmium Concentration. Soil samples were digested with a microwave digester (MWD-600, China)

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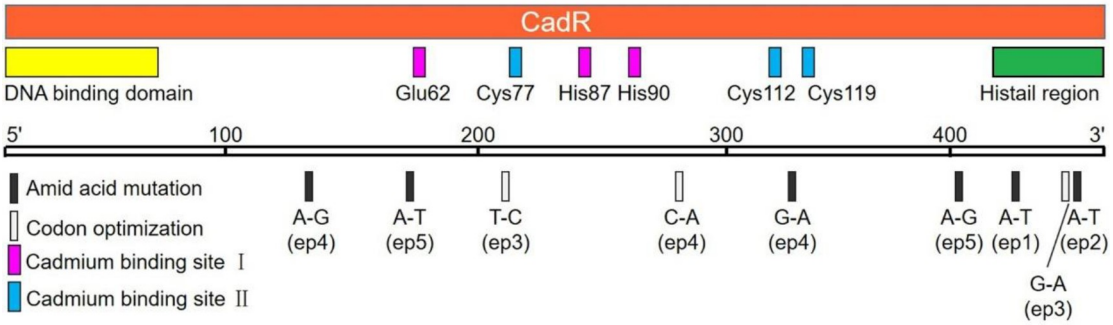

Figure 2. Schematic mutation-site summary of the CadR mutants; ep1 (epCadR1) to ep5 (epCadR5) are the first to last round of evolution mutants.

to determine the elements. The soil sample digestion was carried out using the way described in Text S4 of the Supporting Information (HJ 803-2016). Total cadmium in soil and water samples was determined by iCP Qc ICP-MS (Thermo Fisher Scientific, MA, USA).

Application of Evolved Cadmium-Responsive Bacteria. The standard curve was constructed using a cadmium standard solution from the National Institute of Metrology (Beijing, China). Eight cadmium concentration standards ranging from 0 to 8 μg/L were used to generate the cadmium-induced fluorescence response. The storage solutions of environmental samples were diluted in a certain proportion and then treated with the evolved bacteria in the same way, and the cadmium concentration was calculated according to the standard concentration curve. For fluorescence observation, 0.2 g of the treated soil was directly added to the bacterial liquid in the logarithmic growth phase, and the fluorescence of *E. coli* was observed with a Leica DM IL LED inverted fluorescence microscope after 2 h of culture.

Statistical Analysis. Flow cytometry data were analyzed with FlowJo software (v. 10.0). Statistical analysis was performed with GraphPad Prism 8.0 (GraphPad Software). Nonlinear curves and standard curves were fitted by the Hill equation (four parameters) and linear regression, respectively. All experiments were repeated three times, and all values were expressed as the arithmetic mean ± standard deviation (S.D.). Comparisons between multiple group means were performed using one-way analysis of variance (one-way ANOVA), and a t test was used to assess the significance level, with $P \leq 0.05$ considered statistically significant.

RESULTS AND DISCUSSION

Construction and Directed Evolution of Wild-Type Cadmium-Responsive Bacteria Biosensor. Cadmium resistance (CadR) protein from the cadmium-responsive transcriptional regulator (MerR) family can bind to Cd(II), which was characterized from the environmental isolate *Pseudomonas aeruginosa* BC15.[28] We constructed the wild-type cadmium-induced bacteria pSB1k3-CadR-Kana-GFP by cloning the cadmium-responsive CadR gene and its specific promoter pCadR into the expression plasmid pSB1k3-mAID-Kana-GFP (Figures S2 and S3). As shown in Figure 1a, CadR protein performs its function as a dimer, and dimerization is required for activity and structural integrity. The constitutively expressed CadR protein binds to the promoter CadR (pCadR), which results in a conformational change of the pCadR duplex and represses the protein expression of GFP. CadR−cadmium binding is evident in the presence of cadmium, which causes promoter DNA more easily to be recognized by RNA polymerase and leads to GFP expression. Wild-type pSB1k3-CadR-GFP was used as the original template to introduce random mutations into the protein coding region using ep-PCR to construct a mutant library. After FACS sorting, the mutants with the highest fluorescence compared to the template bacteria were retained and used as templates for the next round of evolution.

The five-round evolved mutant library and the fluorescence response of the screened mutant compared to the template are shown in Figure 1b. Each bacteria mutant library was characterized through a cadmium induction test. With increasing rounds of evolution, the cadmium concentration decreased to obtain mutants with higher fluorescence response to low-concentration cadmium. The mutant library without cadmium induction was selected as the negative control. A right-shifted peak on the flowchart was generated due to the fluorescence response of evolved mutants, which was defined as a positive zone of the induced library. Then, approximately 1% of the mutants in the positive zone were picked up. After verification with cadmium induction, the mutant with the highest fluorescence response intensity was sequenced and selected as the template for the next round of evolution. The fluorescence responses of the optimal mutant obtained in rounds 1−5 were improved by 48.8%, 25.5%, 44.7%, 33.2%, and 50.6%, respectively, compared to the template. When mutants from the fifth round screening generated significantly improved fluorescence induced by cadmium at the concentration of 5 μg/L (Chinese national quality standards for drinking water), the directed evolution of cadmium-responsive bacteria was completed.

Sequence Analysis of the Mutation Sites on CadR Gene Coding Sequence. Sequence alignment was performed with ClustalW and GeneDoc, and the mutation sites on the CadR coding sequence were marked (Figure S4). CadR from *Pseudomonas aeruginosa* contains 156 amino acids. Cadmium-binding site I composed of cysteines (Cys77, Cys112, and Cys119) among *Pseudomonas* species and strains is conserved. Metal binding site II located in the middle of the protein consisted of two histidines (His87 and His90), a glutamate (Glu62), and a variable final ligand Histail.[29] It was predicted that the histidine-rich C-terminal of CadR might be related to the metal specificity of the cadmium operon.[30] The fifth round mutant epCadR5 had a total of nine base mutations, of which three mutations were codon optimized, and the other six sites resulted in amino acid mutations (Figure 2, Figure S5). None of the mutations occurred in the conserved sites of CadR, and thus, the ITC results only

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showed a 1.5-fold increase in the cadmium binding affinity of epCadR5 protein compared to wtCadR rather than an exponential increase, which further indicated that these conserved sites were mainly responsible for the binding of cadmium (Figure S6). There were two amino acid mutations (His 147 Leu, His 152 Leu) in the coding region of the second round CadR mutant (epCadR2), which were located at the C-terminal His-tail region. Histail could inhibit the cadmium binding of CadR when transforming from a disordered state to a loop state and packing in the center of the CadR homodimer. Therefore, the flexible His-tail region conferred the switching between the "on" and "off" states.[29] His 147 Leu and His 152 Leu reduced the background fluorescence and enhanced the fluorescence response to cadmium. Cadmium binding site I was an allosteric site. Cys119 moved toward the coordination center with the presence of cadmium, and Cys77 became ordered and coordinated with the cadmium ion. Conserved residues are important for the structure of MerR family proteins, and mutations in these conserved residues will cause structural and functional changes. However, accumulated mutations at noncritical sites can also bring about changes in protein properties. Hakkila et al. obtained a series of mutants with response to cadmium by directed evolution of a mercury-responsive protein, and the results discovered that accumulating unrelated mutations in noncritical sites eventually brought about a great change in the overall performance of the protein.[31] The fourth round of amino acid mutation between Cys112 and Cys119 might stabilize the conformation recognized by cadmium. Cadmium binding site II further altered the conformation of the DNA binding domain as an additional allosteric site, and this function was realized by the strong coordination bond between Glu62 and His87/His90. The amino acid mutation near the critical sites might lead to a change in the stability of this specific conformation.

CadR is critical for detection as a core component of the bacterial biosensor. The expression of CadR derived from *Pseudomonas aeruginosa* may differ from *E. coli* as there is a codon bias between different bacteria genus.[32] We did not explore the role of each base mutation in the detection, but we did not rule out the possibility that some base mutations were CadR codon optimized, making the expression of CadR suitable for the needs of cadmium detection. It was most obvious that there were only base mutations in the third round of mutations, and the response of the bacteria to cadmium was significantly enhanced even in the absence of amino acid mutations.

Analytical Performance of the Evolved Bacteria for Cadmium Assay. An ideal bacterial biosensor should possess not only the advantages of high sensitivity and specificity of chemical analytics but also the ability of rapid and anti-interference detection.[33] Therefore, the dose-dependent effect, time-dependent effect, and the specificity of the evolved bacteria were investigated.

Dose-Dependent Response to Cadmium. To validate the dose−response relationship of the evolved bacteria, the wild-type and the evolved bacteria were incubated at 37 °C for 2 h in cadmium standard solutions within gradient ranges. The data showed that the two bacteria produced GFP fluorescence signals with a dose-dependent effect relationship (Figure 3). The relative fluorescence intensity in the epCadR5 bacteria to 15 μg/L cadmium increased from 5.1 to 8.5, an improvement of 1.67-fold. The limit of detection (LOD) was calculated using the formula LOD = $3S_a/b$, where S_a represents the

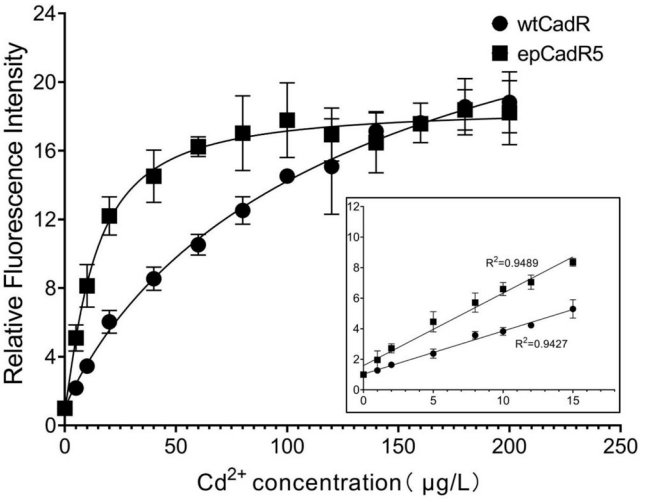

Figure 3. Dose-dependent response of bacterial biosensors to cadmium in *E. coli* strain JudeI. R^2 values resulted from linear interpolation of the averages from three replicates.

standard deviation (SD) of the blank, and b represents the linear slope.[34] The LOD of wild-type CadR bacteria was 1.15 μg/L, while the evolved epCadR5 bacteria were reduced to 0.45 μg/L in the water matrix. So, the sensitivity of biosensor bacteria was improved by directed evolution. The detection limits of the recently reported cadmium whole-bacterial biosensors constructed using CadR or CadC were mainly concentrated around the cadmium limit standard concentration (5 μg/L), for example, 1.12 μg/L for Jia et al.[20] and 9 μg/L for Bereza-Malcolm et al.[35] The LOD of our evolved epCadR5 bacterial biosensor was also much lower than the cadmium limit standard concentration, which was important for the detection of trace amounts of cadmium in samples. We also verified the gradient concentration of the cadmium response for each round of mutants (Figure S8). Overall, these results suggested that the detection performance of the evolved mutants was better than that of the wild-type bacteria.

The background leakage of GFP expression and the fluorescence values of wtCadR and epCadR5 bacteria at different concentrations are shown in Table S4. Despite the higher background fluorescence leakage of epCadR5, it also produced significantly higher fluorescence intensity increments in response to cadmium than wtCadR bacteria. After replacing the reporter system with sfGFP, we found an overall increase in the fluorescence intensity of the bacteria in response to cadmium, which provided us with ideas for subsequent modifications to the reporter gene module (Figure S7).

Time-Dependent Response to Cadmium. The response time of bacterial biosensors to specific pollutants is considered as an essential indicator of their performance.[36] Given this, we performed a time-response assay under the cadmium concentrations of 5 and 50 μg/L with the gradient induction time ranging from 30 to 240 min. The wild-type and epCadR5 bacteria were incubated in two concentrations of standard cadmium solution for different time spans to represent the time-dependent effect of the bacteria, and the results are shown in Figure 4. Under the cadmium concentration of 5 μg/L, epCadR5 exhibited a more than 3-fold fluorescence increase compared with the control within 30 min, and the highest fluorescence expression was approximately twice that of wtCadR. Both bacteria maintained the same fluorescence

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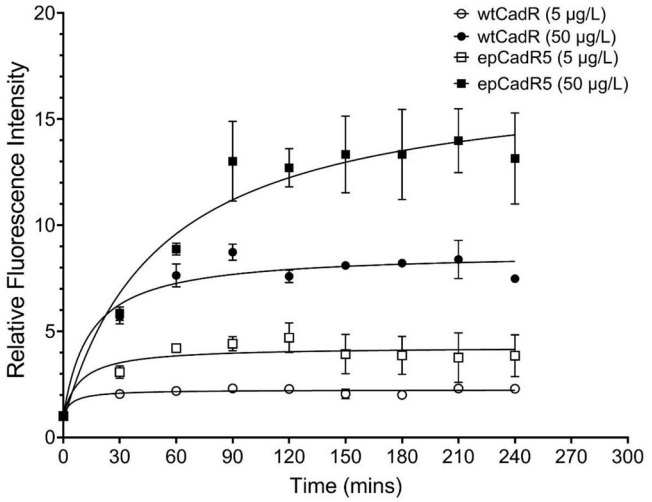

Figure 4. Time-dependent response of bacterial biosensors to cadmium in *E. coli* strain JudeI. Error bars resulted from linear interpolation of the averages with three replicates.

response trend at the cadmium concentration of 50 μg/L, and the final fluorescence intensity of epCadR5 was also close to twice that of the wild-type bacteria. The results indicate that the fluorescence change of the evolved cadmium-responsive bacteria was more significant than that of the wild-type and the significant fluorescence increase could be determined within 30 min, which was conducive to the rapid detection of cadmium contamination.

Specificity of Evolved Bacteria. In addition to the sensitivity and intensity of the signal output, the specific response to cadmium is also an essential criterion for verifying cadmium-responsive bacterial biosensors. As the cadmium binding sites of the wild-type CadR protein can bind to a variety of heavy metals, such as cadmium, lead, and zinc, the wild-type bacteria generally has the disadvantage of poor specificity.[37] To this end, we verified the cadmium specificity of evolved bacteria using several other heavy metals that are usually associated with cadmium contamination.[38] The specificity of the biosensor was defined as the ratio of the fluorescence intensities of cadmium at the concentration of 0.1 μM to that of other heavy metals at the concentration of 10 μM.[9] The mass concentrations of heavy metals corresponding to each of the four columns are shown in Table S5 of the Supporting Information. As shown in Figure 5, the relative fluorescence intensity of wild-type CadR bacteria increased 6 or 12 times after induction with the same molar concentration of Pb or Zn, while the fluorescence response intensity of evolved epCadR bacteria to Pb and Zn at the same concentration decreased to 4 and 3.5, respectively. It was calculated that the specificity of wtCadR was 0.29, while the specificity of epCadR5 was 1.97, which was 6.8 times higher than that of wtCadR. As a congener of cadmium, the response of the evolved bacteria to mercury increased along with the response to cadmium during directed evolution, but only 2-fold fluorescence enhancement at a mercury concentration of 200 μg/L could be detected. Furthermore, epCadR5 exhibited improved anti-interference ability toward the mixed heavy metal system (Figure S9). These results suggest that using cadmium as the exclusive inducer to screen the mutant library enhanced the specificity of the bacteria for cadmium.

Application of Bacteria epCadR5 to Detect Cadmium in Real Environmental Samples. As there are abundant microorganisms and a wide range of pollutants in actual environmental samples, the stability of the bacterial biosensor in a complex environment is a critical index related to its ecological application prospect.[39,40] In the actual pollution detection, on-site qualitative tests of soil and water to determine whether the contamination exists are first needed. Second, quantitative analysis with standard methods for sample processing to detect the actual concentration of contamination should be performed. For these two different application scenarios, we first treated the evolved bacteria directly with dried soil particles and qualitatively analyzed the soil samples with possible cadmium contamination by observing the GFP expression through fluorescence microscopy. Then, we quantitatively detected the total cadmium content in soil samples using evolved bacteria epCadR5 and compared the results with ICP-MS. Moreover, cadmium in the natural environment can form complexes or chelates with various components, such as phosphate, which is hard to be utilized by organisms and has no biological toxicity.[41] So, the detection of bioavailable cadmium but not the total cadmium sometimes is more important for health risk assessment. To validate the applied potential of the evolved cadmium-responsive bacteria in the detection of actual environmental samples, water and soil extract samples were also analyzed using the evolved bacteria epCadR5.

Qualitative Assay of Cadmium with Evolved Bacteria epCadR5 in Actual Soil. The soils collected from four representative sites were selected as the test object, and the evolved bacteria epCadR5's ability of rapid detection of actual soil was verified by directly cotreating the bacteria with ground soil. First, the response of the bacteria epCadR5 to a standard cadmium solution was detected. Figure S10 shows that there

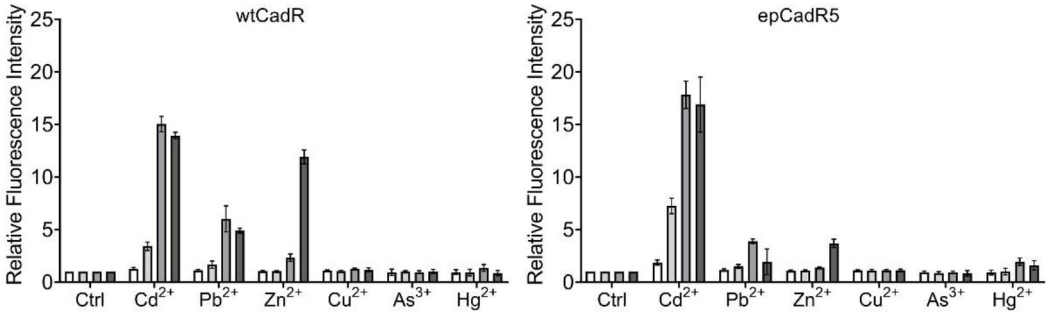

Figure 5. Specificity test of cadmium-responsive bacterial biosensors. Five other pollutants often associated with cadmium-contaminated soil were used to verify the specificity of the evolved bacteria.

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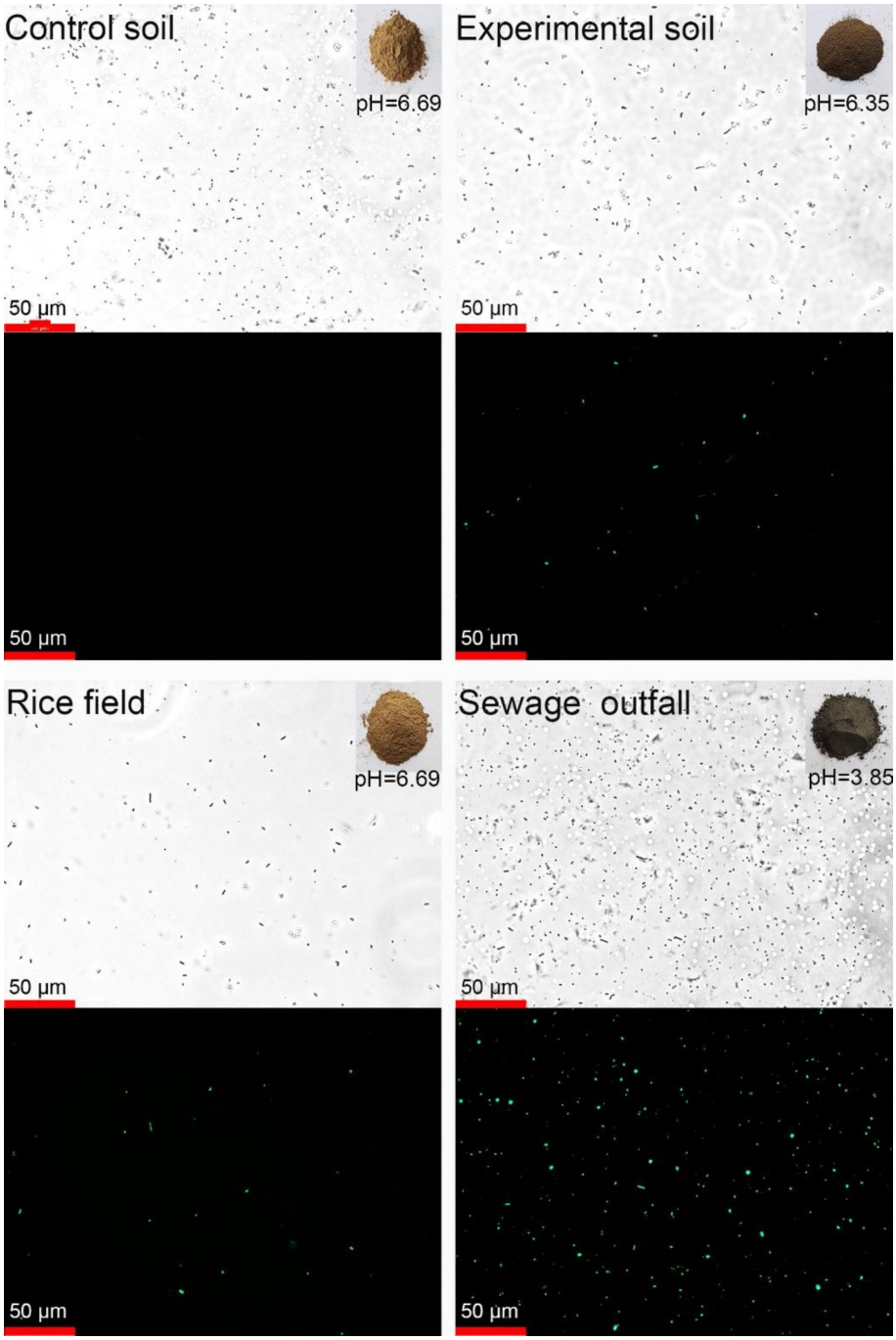

Figure 6. Green fluorescence image of the bacteria cotreated with cadmium-containing soil. pH value was measured in the bacterial solution mixed with soil.

was no GFP fluorescence observed in the control group, which indicated that the background fluorescence expression of the evolved bacteria was low. In cadmium-exposed groups, the fluorescence proportion and intensity increased in a dose-dependent manner. Then, the fluorescence of epCadR5 in actual soils was imaged. As shown in Figure 6, a significant fluorescence expression was observed in all the soils except the control soil, especially in the sewage outfall of the mining area, which indicated that the complex components unprocessed in the actual soil matrix would not affect the work of the bacteria. According to these results, the developed bacteria possess the ability to detect the bioavailability of cadmium and can be applied to on-site qualitative analysis for the presence of contamination.

Quantitative Detection of Cadmium with Evolved Bacteria epCadR5 in Actual Environmental Samples. ICP-MS and the evolved bacteria epCadR5 were used simultaneously to determine the total cadmium concentration in soil samples treated with aqua regia from the experimental field and the mining area in Tongling City. At the same time, to reflect the cadmium concentration in soil bioavailability, the ultrasonic soil extract of the experimental field and sewage outfall were also measured with ICP-MS and the epCadR5 bacteria. The fluorescence−cadmium concentration standard curve was generated using the standard cadmium solution within the

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concentration range of 0−8 μg/L, and a good linear response to the cadmium of epCadR5 was generated (Figure 7a). To

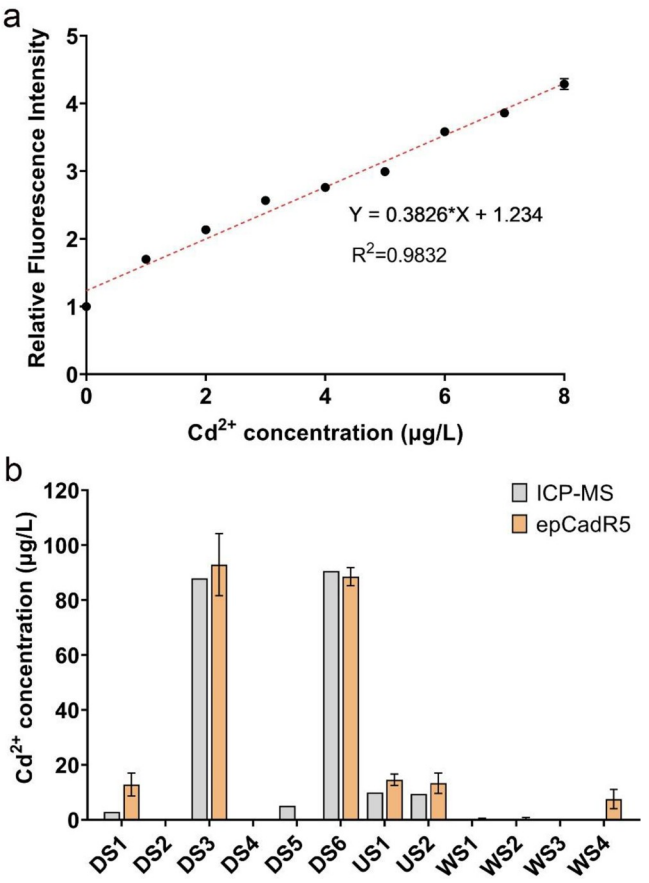

Figure 7. (a) Standard curve of epCadR5 bacteria fluorescence response to cadmium in the concentration range of 0−8 μg/L. Concentration calculated from hyperbolic slope ($y = 0.3826x + 1.234$, $R^2 = 0.9832$). (b) ICP-MS and epCadR5 cadmium-responsive bacteria detection of actual environmental samples. DS1−DS6 represent the digestion solutions for reservoir, sewage outfall, experimental field, control field, downstream, and rice field soil, respectively. US1 and US2 represent the sewage outfall and experimental field soil ultrasonic extract, respectively. WS1−WS4 represent water samples from downstream, reservoirs, sewage outfall, and rice fields, respectively. Dilution of samples: DS2 and DS3 were diluted 40 times. DS1, DS4, and DS5 were diluted 20 times. US1, US2, WS1, and WS2 were diluted five times.

obtain the actual cadmium concentration values in the linear range of the standard curve, gradient dilutions of different concentrations were carried out on each sample, and an optimal dilution ratio was selected for the actual detection.

The results of the total cadmium content in tested soil samples are shown in Figure 7b. The epCadR5 bacteria detected cadmium only in the digest solution of the reservoir, experimental field, and rice field, and the cadmium concentrations calculated from the standard curve were 12.85, 92.93, and 88.53 μg/L, while the results of ICP-MS were 2.92, 87.88, and 90.50 μg/L, respectively. Neither ICP-MS nor epCadR5 detected the cadmium concentration in the sewage outfall soil digest, and the spiked recovery of ICP-MS in detecting this digest was only 5.7%. Since the sewage outfall soil was collected from the mine area of pyrite, which indicated that there was some interfering element that seriously interfered with the detection of cadmium after the aqua regia digestion, the epCadR5 detection results found that the growth state of bacteria was greatly affected when using the sewage outfall soil digest as an inducer. It is possible that the soil at this site contains extremely high concentrations of some other heavy metal elements, and the toxic effects enhanced by the aqua regia digestion caused the bacteria to fail to produce fluorescent protein normally. The same problem was found for the downstream soil digestion solution detection. Although the ICP-MS results showed a concentration of 5.09 μg/L, the recovery was still only 19.7%, while epCadR5 failed to detect cadmium. In general, epCadR bacteria provided similar results to ICP-MS for total cadmium analysis, while for soils of complex composition even the use of ICP-MS cannot eliminate the interference of complex components.

The absolute bioavailability can be reflected using bacterial biosensors.[42] The cadmium assays of soil ultrasonic extract and water samples reflect the bioavailable cadmium in the real samples (Figure 7b). For water samples, cadmium was only detected in the rice field with a concentration of 7.58 μg/L. No cadmium was detected in the other surface water samples collected around the mining area. The detection results of epCadR5 and ICP-MS on ultrasonic water samples showed that the evolved bacteria can provide a reference for bioavailability, and its measured cadmium concentration was close to that of ICP-MS.

Overall, the detection results of cadmium concentration in detectable samples using the evolved bacteria were close to those of ICP-MS, which indicated that the evolved bacteria had the potential for assessing the cadmium concentration in actual environmental samples. On the other hand, with the anti-interference ability, the evolved bacteria possess the potential to be a quantitative biosensor for the determination of bioavailable cadmium.

In this study, we successfully improved the performance of wild-type cadmium-responsive bacterial biosensor with high-throughput screening, and the evolved bacteria were also successfully applied to the detection of actual environmental samples. The estimated cadmium concentration values detected by the evolved bacteria were close to the results provided by ICP-MS. In addition, the excellent performance of the evolved bacteria in the detection of actual environmental contaminated soil without pretreatment offers the possibility of its application for direct and rapid detection of more contaminated samples. The successful application of directed evolution combined with high-throughput screening to improve the performance of bacterial biosensors has provided a reliable idea for the development of more high-performance bacterial biosensors for certain pollutants.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c00627.

Methods and results, construction of bacterial biosensor, fluorescence activated cell sorting, digestion of environmental soil samples, purification of wtCadR and epCadR proteins, isothermal titration method for determining the affinity of CadR proteins, cadmium response for each round of mutants, distribution of sampling sites, map of original CadR sequences and their alignment results, map of ITC test for wtCadR and epCadR

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proteins, bacterial cadmium response diagram for replacing different reporter systems, fluorescence response diagram for evolved bacteria in the composite system, microscopic observation diagram for bacteria treated with standard samples, table for primer designing, table for wtCadR and epCadR5 biosensor cadmium-response fluorescence value, and heavy metal concentration used for specificity detection (PDF)

AUTHOR INFORMATION

Corresponding Author

Shaopeng Chen − *Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei 230031, China; University of Science and Technology of China, Hefei 230026, China; School of Public Health, Wannan Medical College, Wuhu 241002, China;* orcid.org/0000-0002-3632-9727; Phone: +86-0551-65593096; Email: spchen035@ipp.ac.cn

Authors

Yeshen Cai − *Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei 230031, China; University of Science and Technology of China, Hefei 230026, China*

Kaili Zhu − *Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei 230031, China; University of Science and Technology of China, Hefei 230026, China*

Liang Shen − *Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei 230031, China; University of Science and Technology of China, Hefei 230026, China*

Jie Ma − *School of Public Health, Wannan Medical College, Wuhu 241002, China*

Lingzhi Bao − *School of Public Health, Wannan Medical College, Wuhu 241002, China*

Dongdong Chen − *Institute of Environmental Physics and Technology, Anhui University, Hefei 230039, China*

Liangchen Wei − *School of Public Health, Wannan Medical College, Wuhu 241002, China*

Nan Wei − *Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei 230031, China; University of Science and Technology of China, Hefei 230026, China*

Binmei Liu − *Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei 230031, China; University of Science and Technology of China, Hefei 230026, China*

Yuejin Wu − *Hefei Institutes of Physical Science, Chinese Academy of Sciences, Hefei 230031, China; University of Science and Technology of China, Hefei 230026, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.2c00627

Notes

The authors declare no competing financial interest.

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