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Chromosomal expression of CadR on *Pseudomonas aeruginosa* for the removal of Cd(II) from aqueous solutions

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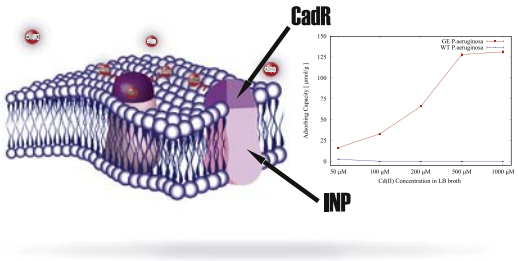
HIGHLIGHTS

- We first express CadR that is specific to cadmium on the surface of *P. aeruginosa*.
- The chromosomal engineering *P. aeruginosa* exhibiting excellent adsorption capacity
- The chromosomal engineering *P. aeruginosa* has great hereditary stability.
- The engineering *P. aeruginosa* is expected to be used in practical applications.

GRAPHICAL ABSTRACT



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ABSTRACT

Genetically engineered bacteria for pollution control of heavy metal have been widely studied, however, using *Pseudomonas aeruginosa* (*P. aeruginosa*) that can adapt to various circumstances to remediate heavy metal pollution is rarely reported. In this study, we employed CadR, a cadmium (Cd)-specific binding protein, displaying on the surface of *P. aeruginosa* with chromosomal expression. The genetically engineered (GE) *P. aeruginosa* still flourished in the 30th generation in the LB broth which contained 100 μM Cd(II), exhibiting an excellent genetic stability. Chromosomally expressed *P. aeruginosa* showed an adsorption capacity of up to 131.9 μmol/g of Cd(II). In addition, the low concentration of the coexisting two valence ions has no significant effect on adsorption capacity of Cd(II). This study provides a direction for application of *P. aeruginosa* in environment remediation.

1. Introduction

Cadmium (Cd) has been used in industries for a long period of time (Baldwin and Marshall, 1999; Ming et al., 2016), and still plays a critical role in modern industries, such as PVC production, nickel-cadmium battery manufacturing, printing and dyeing, nuclear power plants, and others. Although some Cd-containing products can be recycled, a large proportion of cadmium pollution is caused by dumping and incinerating cadmium-polluted waste. In addition, the use of phosphate fertilizers in agriculture brings severe cadmium pollution burden to the environment. These lead to the wide occurrence of cadmium pollution in China (especially the paddy soil of South China) and the production of 'cadmium rice' in the polluted farmland. As reported, more than 10% of rice grain samples across China exceed the allowable cadmium standard of 0.2 mg/kg (Cui et al., 2017). Heavy metal pollution in a variety of environment has been a severe threat to the human's health. Therefore, it is an urgent demand to seek for an efficient and environmentally compatible

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method to remove or detoxify heavy metals (Xu et al., 2012b; Chen et al., 2016; Song et al., 2017). Currently, the pollution treatment technologies contain physical (Gong et al., 2009; Xu et al., 2012a; Ren et al., 2017), chemistry (Long et al., 2011; Tang et al., 2014; Tan et al., 2015) and biological methods (Wu et al., 2017; Yang et al., 2010; Cheng et al., 2016). Heavy metal remediation through common physico-chemical techniques is expensive and unsuitable in a case of voluminous effluents containing complicated contents. Therefore, biotechnological approaches that are designed to cover such niches have received a great deal of attention in the recent years (Malik, 2004), and these approaches usually provide a complete clean-up method for heavy-metal without secondary pollution (Huang et al., 2003).

In the natural environment, some microorganisms can resist the invasion of heavy metal ion. They have five major strategies to resist heavy metal damage. 1, Reduce absorption and keep the heavy metal content in the microorganism cells at a very low level without causing a toxic effect on the cells. 2, Some microbes can drive the cell's heavy metal ions out of the cell in an active way, so as to maintain a low level of content in the cells. 3, Redox action. 4, In the extracellular domain, a combination of toxic heavy metal ions can be combined (including cell surface adsorption), thereby reducing the concentration of environmental toxic heavy metals and achieving the purpose of detoxification. 5, Adsorbing heavy metal ions to the special structures on microorganism cells surface. Under the severe specific heavy metal ion pollution, like serious Cd(II) single pollution, display of a specific heavy metal binding protein on cell surface for adsorption is a feasible way. *P. aeruginosa* is a ubiquitous bacterium which can be isolated from different habitat and has a preeminent environmental adaptability. In general, *P. aeruginosa* was used for the expression of rhamnolipid, biofilm research and clinical medicine research. In some species of *Pseudomonas* bacteria, *P. stutzeri* and *P. putida* were used as carriers of genetic engineering to control heavy metal pollution. We chose *P. aeruginosa* as the recipient in this study. The protein expression in bacteria includes plasmid expression and chromosome expression, whose method is suitable for *P. aeruginosa* also in view.

Compared with chromosome expression, the plasmid expression of polypeptide or protein is widely applied because of its convenience and efficiency, but, the low hereditary stability of plasmid expression is the bottleneck of practical application. Increased understanding of microbial genomes and proteomes in recent decades, along with advances in recombinant technology, has significantly improved our ability to manipulate microorganisms in biotechnological applications (Chen et al., 2015; Zhang et al., 2015). In particular, the ability to express heterologous proteins on the cell surface has become the foundation of a wide range of important medical and environmental applications (Smith, 1985).

Therefore, considerable effort should be made in revealing the Cd(II)-specific engineering bacterium. Displaying a Cd(II)-specific protein on the bacterium surface is a feasible way. CadR is a Cd(II)-responsive MerR homologue Cd(II)-binding protein first isolated from the rhizobacterium *Pseudomonas putida 06909*. It can regulate the cellular Cd(II) concentration by regulating the expression level of CadA, a Cd(II) efflux ATPase, CadR contains 147 amino acids and three domains: the DNA binding domain, the metal binding domain and the coupling domain (Lee et al., 2001; Brocklehurst et al., 2003; Brown et al., 2003). Three cysteine residues (Cys 77,112, 119) and its histidine rich C-terminus are predicted as possible Cd(II) binding sites. The sensitive and specific recognition of Cd(II) by CadR has been developed as Cd(II) sensors (Wu et al., 2009; Chiu et al., 2013; Tao et al., 2013). To anchoring the CadR on the surface of *P. aeruginosa*, a stable anchoring protein is essential. The ice-nucleation protein (INP), an outer membrane protein from *Pseudomonas syringae*, is capable of catalyzing the formation of ice in supercooled water (Wolber et al., 1986). All identified INPs comprised three distinct structural domains distinguished as the N-terminal domain (15%), the C-terminal domain (4%) and the central repeating domain (81%) (Li et al., 2012). The INP N-terminal domain, which contains three or four transmembrane spans and is responsible for targeting to the cell surface (Kozloff et al., 1991; Schmid et al., 1997), can be used to display foreign proteins on the *Escherichia coli* cell surface (Fan et al., 2011; Khodi et al., 2012; Li et al., 2009). The full-length INP is quite large (1200–1500 aa) and neither the C-terminal domain nor the central repeating domain (CRD) of the INPs harbors signal peptide sequences (Li et al., 2012). Therefore, the INP-N (the N-terminal domain) or the INP-NC (containing only the N-terminal and C-terminal portion) was usually used as the anchoring motif to direct translocation of foreign proteins onto the cell surface or the cell periplasm (Khodi et al., 2012; Chungjatupornchai and Fa-aroonsawat, 2009).

In this study, we employed N-terminal domain of INP (*inaXN*) as the anchor and fuse *cadR* with inaXN, and then insert it in the chromosome of *P. aeruginosa* by tripartite mating. This is the first use of *P. aeruginosa* for the surface expression of specific heavy metal binding proteins. Engineered *P. aeruginosa* exhibit excellent selectivity to Cd(II) and expression stability. Furthermore, admirable Cd(II) adsorption capacity of GE *P. aeruginosa* was measured in laboratory solution environment. Further application experiments of Cd(II) bioremediation by engineered *P. aeruginosa* could be expected.

2. Materials and methods

2.1. Source of INP-cadR

The coding genes of INP (GenBank: FJ797399.1) and CadR (Gene ID: 1186965) were contained in plasmid pUC57, and synthetized by GENEWIZ. The primers used in this study were synthetized by GENEWIZ. The *INP* and *cadR* were conjugated through touchdown-PCR: at first, Primer 1 (5'-AAGCTCTAGAGCATGAA CCGCGAGAAGG TGCTGGCCCT-3' (XbaI site underlined)) and Primer 2 (5'-GCCA GCTCGCCGATCTTCATGAACTCGATCTCGCGGGTG-3') were employed to amplify the INP coding gene; Primer 3 (5'-CACCCGCGAGATCGA GTTCATGA AGATCGGCGAGCTGGC-3') and Primer 4 (5'-AATAT CGGGATCCCGGTGGCC GTGGCTGCGG CCCACGT-3' (BamHI site underlined)) were employed to amplify the *cadR* coding gene. In overlap PCR process, Primer 1 and Primer 4 were utilized to amplify the fused INP and *cadR* gene. The overlap PCR profile was as follows: 95 °C for 5 min; 2 cycles of 94 °C for 30 s, a bring down temperature for 30 s, and 72 °C for 45 s, which was from 72 °C to 55 °C (0.5 °C each time), and then, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and a final elongation at 72 °C for 10 min. The amplification product was named *INP-cadR*.

2.2. Construction of target plasmid

The pET-28a-c(+) (pET28a for short) plasmid vector was donated by Prof. *Yanyang Wu* (Hunan Agricultural University). The plasmid pET28a and *INP-cadR* fragment was digested by restriction enzyme BamHI and XbaI, successively. The enzyme-digested products were purified by DNA Purification Kit followed. The purified *INP-cadR* fragments were ligated into the digested pET28a that used to provide efficient T7 promoter, the putatively integrated new plasmid was named pET28a-*INP-cadR*. Primer T7-F (5'-GGACTAGTCAGATCTCGATCCCGCGAA ATT-3' (SpeI site underlined)) and T7-R (5'-CCTTAATTAACAAAAAACCCCTC AAGACCC G-3' (PacI site underlined)) were employed to amplify and confirm the T7 promoter fragment that contain the *INP-cadR*, named pET-*INP-cadR*. Then a new round of restriction enzyme digestion of the artificial template was performed. The pET-*INP-cadR* fragment and plasmid pBAM1 digested by restriction enzyme SpeI and PacI were performed in sequence; the enzyme-digested product was purified by

DNA Purification Kit (TIANGEN). The digested pET-*INP-cadR* fragment was ligated into digested pBAM1 in sequence, generating a new putatively plasmid, named pBAM1-pET-*INP-cadR* (pBAM1-*cadR* for short) (Fig. 1). The pBAM1-*cadR* was transformed to *E. coli BW23473* competent cell that could manufacture λpir protein. All restrictive endonucleases were purchased from NEB.

2.3. Display of CadR on P. aeruginosa

The plasmid pBAM1-*cadR* was delivered from *E. coli BW23473* into *P. aeruginosa* (ATCC: 9027) via triparental mating with the assistance of the helper strain *E. coli EB167* (PRK2073). The bacteria's cultures were cultivated to the midexponential phase, then mixing these three bacteria in the ratio of 1:1:1(v/v/v) (500 μL). After centrifugation at 5000 rpm for 5 min, the mixture was washed with PBS buffer (pH=7.4) twice. Then mixture was incubated at 30 °C for 48 h by tripartite mating on membrane filters (0.22 μm) that flooring on antibiotic-free LB agar plates (Chen et al., 2014). The membrane filter was washed by sterile PBS buffer (pH=7.4). The eluate was inoculated to the selective medium (LB agar plates contain 100 μM cadmium and 50 μg/L kanamycin) in appropriate diluted concentration, then cultured in selective medium at 30 °C for 36 h. The positive colonies were selected and cultivated in Cd(II)-contained LB broth (500 μM) to examine the resistant ability to Cd(II). Putative engineered *P. aeruginosa* that can tolerate high concentration of Cd(II) are preserved. All cadmium concentrations in this study were disposed by cadmium chloride.

Cells were harvested and identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Western-blot analysis was used to probe the expression of CadR. The cells were inoculated to LB broth containing 500 μM Cd(II) and 50 μg/mL of kanamycin, and the cultures were grown to an OD_{600} = 0.5–0.7 after being cultivated overnight at 30°C under gentle shaking. The membrane protein of the engineered *P. aeruginosa* was extracted by Bacterial Membrane Protein Extraction Kit (BestBio). The Western-blot analysis was to probe the expression of CadR (INP-CadR: 35.316 kDa) in engineered *P. aeruginosa* (GE *P. aeruginosa* for short). 10 μL of total membrane protein was loaded per lane and electrophoresed in a 12% SDS-PAGE gel, and the separated proteins were electrophoretically transferred to a polyvinyl difluoride (PVDF) membrane at 200 mA. The PVDF membrane was blocked at 4 °C overnight in TBST containing 5% dry milk and allowed to incubate with anti-His Mouse monoclonal Antibody (CMCTAG) diluted in 1:2000 at room temperature for 2 h. After washing three times with TBST (10 min for once), the blot was incubated with peroxidase-conjugated goat anti-mouse IgG (H+L)-HRP (CMCTAG) at 1:5000 dilution in blocking buffer for 1 h. After washing again in TBST, the immunoreaction was visualized by super ECL detection reagent (Pro-light HPR, TIANGEN).

Genetic stabilization of *cadR* in GE *P. aeruginosa* was also tested. The selected monoclonal GE *P. aeruginosa* was cultivated in 1.0 mL selective LB broth (contained 100 μM Cd (II)) at 30 °C for 12 h by gentle shaking, and then, 10 L of the cultures was added to 1.0 mL fresh selective LB broth for the next round of amplification. Repeating this process until the bacteria cannot grow in selective LB broth any more. In this experiment, the *E. coli BL21(DE3)* bearing pET28a-*INP-cadR* as the control. The OD_{600} that after 12 h gentle shaking of each generation was recorded.

2.4. Resistance assays to Cd(II)

Prior to Cd(II) biosorption, the GE *P. aeruginosa* (harboring pBAM1-*cadR*) and the wildtype *P. aeruginosa* were grown in LB broth at 30 °C overnight until OD_{600} =0.5. The cells were collected after centrifugation at 5000 rpm for 5 min at room temperature, and resuspended in 1 mL PBS buffer and 10 μL cultures before being incubated to LB broth containing 0, 50 μM, 100 μM, 200 μM, 500 μM or 1000 μM Cd(II), respectively. The adsorption experiment was performed at 30 °C for 24 h with 120 rpm shaking. After incubation, the cells were separated from the reaction solution by centrifugation at 5000 rpm for 10 min at room temperature and filtered by 0.22 μm sterile syringe filter. The Cd(II) concentration of reaction solution was measured by flame atomic absorption spectrometric method (AFS). The effective adsorption rates of Cd(II) were calculated by (1-α)×100%, where meant the ratio of mercury concentration in solution after over before the adsorption by the bacteria cells.

2.5. Specificity to Cd(II)

To examine whether expression of *cadR* affects the sensitivity of the *cadR* transgenic bacteria to other divalent metal stresses, we incubated GE *P. aeruginosa* and wildtype *P. aeruginosa* to LB broth containing 100 μM Cd(II), 1 mM Mn(II), 2 mM Mn(II), 50 μM Cu(II), 750 μM Zn(II), 10 μM Hg(II) or 300 μM Pb(II), which are greatly higher than the corresponding metal concentrations in the environment. These cultures were cultivated in 30 °C by gently shaking for 8 h. The OD_{600} of the cultures was monitored. The growth state of *cadR* transgenic bacteria treated by divalent metal was used to realize their responses to other divalent metal stress.

We also investigated the effect of different coexistence divalent ions on GE *P. aeruginosa* adsorption capacity. In 50 μM, 100 μM, 200 μM, 500 μM, 1000 μM Cd(II) LB broth, 10 μM Ca(II), Mg(II), Cu(II), Zn(II), Pb(II) were added, respectively. These cultures were cultivated in 30 °C by gently shaking for 8 h. And the adsorptive capacity of Cd(II) with different divalent ion was detected, the concentration of Cd(II) after biosorption was measured by AFS. All experiments in triplicate.

2.6. The influence of different environmental factors on the Cd(II) adsorption capacity

In order to detect the difference of Cd(II) adsorption capacity of GE *P. aeruginosa* under different pH, we inoculate GE *P. aeruginosa* into different pH solutions. Hydrochloric acid is used to adjust pH of the solution to 3, 4, 5, 6 and 7, respectively. We didn't test pH value

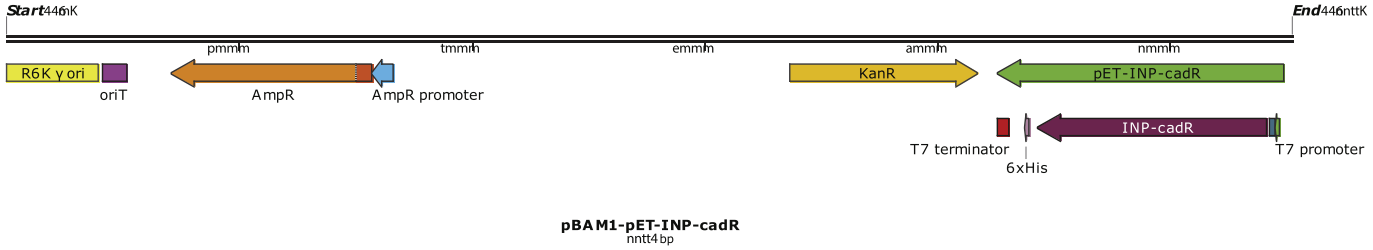

Fig. 1. Schematic representation of the main features of the version of mini-Tn5 engineered in the pBAM1 backbone, containing the CadR coding gene fused with the INP encoding gene, and the *INP-cadR* insert to T7 promoter.

over 7.0, because $Cd(OH)_2$ is the dominant species in solution. The GE *P. aeruginosa* was inoculated to selective medium (contained 100 μM Cd(II) and 50 μg/mL kanamycin) and cultured at 30 °C overnight, and then pick a single colony for amplification in selective broth (contained 100 μM Cd(II) and 50 g/mL kanamycin). 10 μL cultures were inoculated to 20 mL LB broth that contain 200 μM, 500 μM or 1000 μM Cd(II), all concentrations are matching with different pH. The inoculated culture was cultured at 30 °C with gentle shaking for 12 h. After centrifugation (12,000 rpm, 5 min), the supernatant was collected for analyzing the concentrations of Cd(II) by AFS. To investigate the effect of temperature to Cd(II) adsorption capacity of the proposed GE *P. aeruginosa*, we detected its adsorption capacity for different Cd(II) concentration (200 μM, 500 μM or 1000 μM) solutions at different temperatures (0 °C–40° C). Firstly, GE *P. aeruginosa* was inoculated on the selection LB plate at 30 °C for 12 h. Afterwards, monoclonal colony was inoculated to Cd(II)-contained LB broth and incubated at different temperature overnight. The detection method is consistent with the above.

3. Results and discussion

3.1. Chromosomal expression of CadR

Metallothionein (MT) and plant chelating peptides have been widely used for microbiological surface display. They are all rich in cysteine and are not specific to the absorption of heavy metals. When various metal ions exist in the environment, the non-target heavy metal ions will compete with the target heavy metal ions at the metal binding site, the heavy metal ions that have stronger affinity will occupy the binding site. Lead to engineering bacteria adsorption effect of target metal ions is poor, which limits its in the specific application of heavy metal pollution in the environment. We chose CadR as an object to study the expression of specific heavy metal binding proteins in *P. aeruginosa* and chromosomal engineering displaying is a prerequisite to reveal the full potential of CadR in the Cd(II) biosorption. To achieve CadR chromosomal expression, firstly, we assembled the *INP-cadR* coding gene through overlap PCR. The *INP-cadR* coding gene was assembled by overlap PCR, and the product was verified using Primer 1 and Primer 4. The INP coding gene was 537 bp, the CadR coding gene was 444 bp, the existence of 981bp fragment initially confirmed that the overlap PCR was undergone as expected. The pBAM1 has the mini-Tn5 transposon that can randomly insert it's DNA into the bacterial chromosome. The T7 promotor was used to enhance cadR expression. After the *pET-INP-cadR* fragment was inserted into the plasmid pBAM1, the artificial plasmid pBAM1-*pET-cadR* was initially verified by primer T7-F and primer T7-R, original T7 promoter gene was 380 bp, the reconstructed T7 promoter gene was 1341 bp, which account for the *INP-cadR* inserted into T7 promoter (E-supplement). The single restriction digested original pBAM1 and modified pBAM1 by BamHI proved that *pET-cadR* was successfully inserted to pBAM1 (E-supplement).

E. coli BW23473 beared pBAM1-*cadR* was used for donor cell in triple mating with helper strain *E.coli EB167* (PRK2073) and *P. aeruginosa* (as the recipient cell). The donor and the recipient can be distinguished by their different colonial morphology on the selective medium (E-supplement). To examine whether exconjugants had undergone authentic transposition events or resulted from the cointegration of pBAM1-*cadR* into the recipient genome, 12 colonies that resist to Cd(II) (100 μM, on the plate) were chosen, and their sensitivity to higher Cd(II) concentration was examined. Results revealed that several colonies were resistant to 500 μM Cd(II), indicating that the insertion of the mini-Tn5 transposon carried by pBAM1 occurred as expected and the *cadR* had been inserted into *P. aeruginosa* chromosomal genes in the correct orientation with proper reading frame. The *INP-cadR* was displayed on the membrane of the *P. aeruginosa*. The expression of the fusion protein was analyzed by Western-Blot. The total membrane protein of the cell GE *P. aeruginosa* was separated by SDS-PAGE, and the fusion proteins were further verified by immunoblot analysis using anti-FLAG antibodies. The result of Westen-Blot supported the conclusion that CadR protein was displaying on the surface of *P. aeruginosa*. As shown in Fig. 2, the molecular weights of recombinant INP-CadR (calculated MW = 35.316 kDa) were close to their theoretical values.

When exposed to 500 μM Cd (II), *E. coli BL21(DE3)* expressing pET28a-*cadR* grew dramatically greater than *E. coli BL21(DE3)* bearing the pET28a plasmid vector (Fig. 3c), demonstrating that the *cadR* products confer tolerance to Cd(II). In addition, *E. coli BL21(DE3)* bearing plasmid pBAM1-*cadR* was sensitive to 50 μM/L Cd(II). In contrast, *E. coli BL21(DE3)* expressing pET28a-*cadR* can grow in 500 μM Cd(II) broth (Fig. 3b). The *E. coli BL21(DE3)* bearing pET28a-*cadR* shows high tolerance to Cd(II) than *E. coli BL21(DE3)* bearing pBAM1-*cadR*. It could be because that the plasmid pET28a is a multiple copy plasmid, while the plasmid pBAM1 is a suicide plasmid that cannot self-replicate in *E. coli BL21(DE3)* without the λpir protein (Arévalo-Rodríguez et al., 2011).

3.2. Hereditary stability of GE P. aeruginosa

Owing to the segregational instability and structural instability of plasmid vector, the chromosome engineered *P. aeruginosa* (GE *P. aeruginosa* for short) is more stable than *E. coli BL21(DE3)* bearing plasmid pET28a-*cadR* (named *Con E. coli*). Hereditary stability of bacteria was essential for their application in natural polluted environment. To examine whether chromosomal expression has better genetic stability to plasmid expression, Pass-generation assay was implemented to GE *P. aeruginosa* and *Con E. coli*. In the 10th generation, the selective LB broth of *Con E. coli* grown to an OD_{600} under 0.01. In contrast, the GE *P. aeruginosa* still can be alive in the selective LB broth in the 30th generation. Fig. 3d has shown the OD_{600} of selective LB broth after 12 h gentle shaking at 30 °C in the Pass-generation assay. In *Con E. coli* group, the OD_{600} was consistently decreasing to 0.054 (same as blank LB broth), the OD_{600} of GE *P. aeruginosa* was roughly constant in 0.3 –0.5. Therefore, the GE *P. aeruginosa* was the better choice to be applied in nature environment.

3.3. Cd (II) adsorption by surface-engineered cells

To examine whether the GE *P. aeruginosa* (host of pBAM1-*cadR*) was effective to adsorb Cd(II) in the aqueous environment, the GE

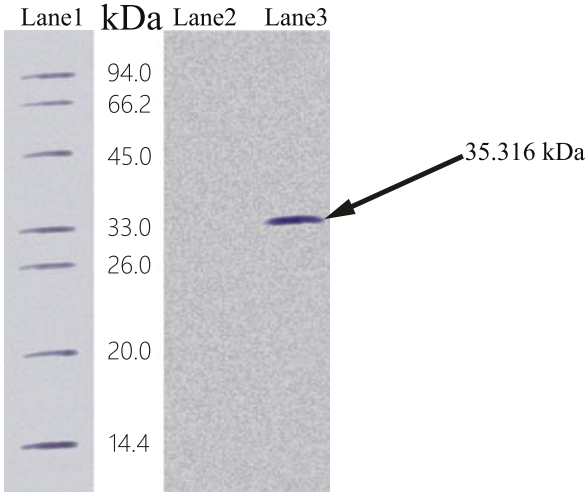

Fig. 2. Western blot analysis of CadR. Lane 1, Protein standards. Lane 2, WT *P. aeruginosa*. Lane 3, GE *P. aeruginosa*.

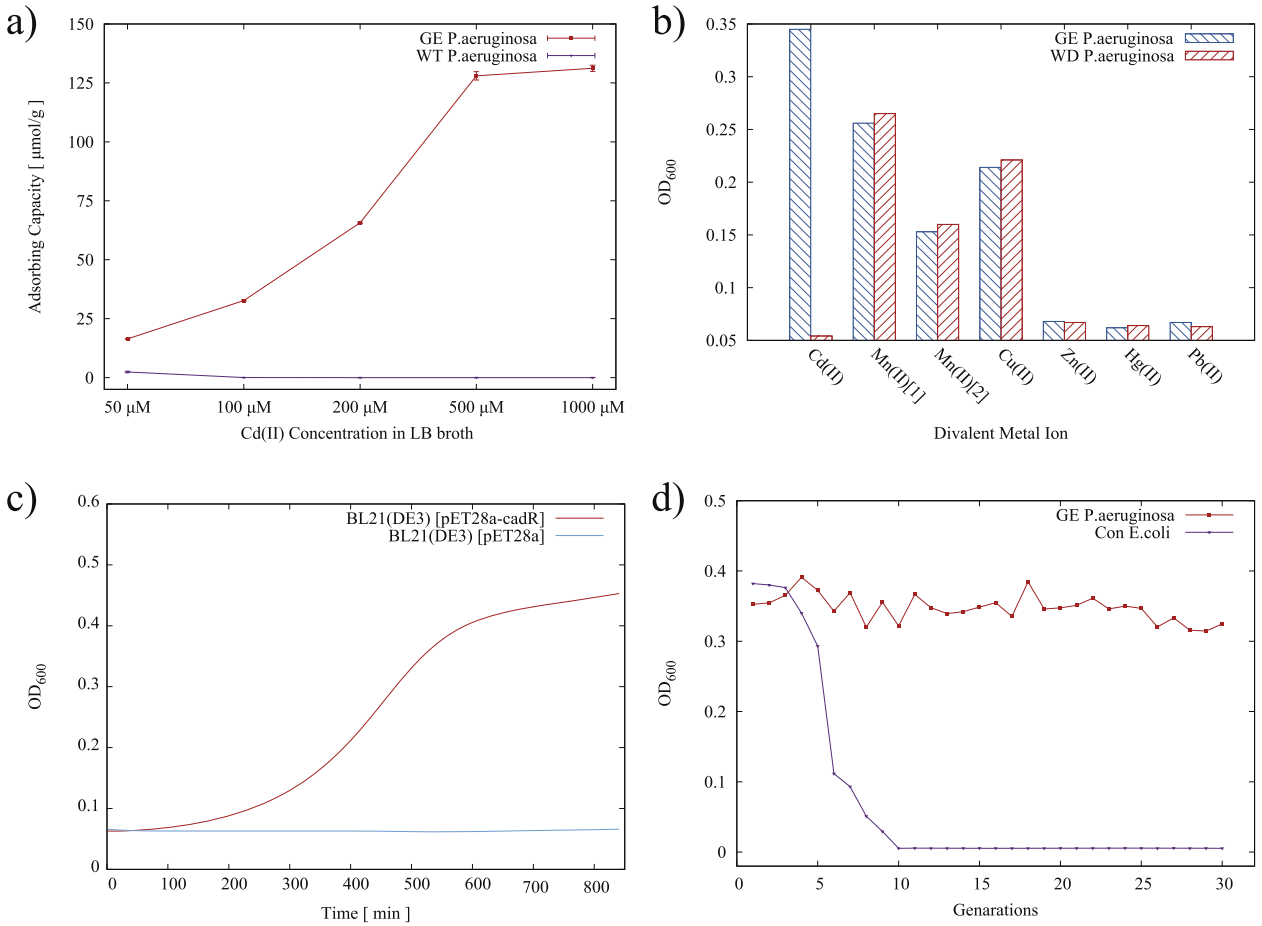

Fig. 3. a) The plasmid pET-*cadR* and plasmid pET28a was transformed to *E. coli BL21(DE3)* competent cells, and its growth curve in LB broth (500 μM/L) was tested. b) The OD600 of GE *P. aeruginosa* when inoculated to LB broth under different divalent metal stress after 8 h gentle shaking. c) Cd(II) adsorption capacity of GE *P. aeruginosa* and WT *P. aeruginosa* in different concentration. d) The OD_{600} of GE *P. aeruginosa* and *Con E. coli* in selective LB broth(500 μM/L Cd(II)) after 12 h gentle shaking in 30°C in the Pass-generation assay.

P. aeruginosa was incubated in the LB broth which contained 50 μM, 100 μM, 200 μM, 500 μM or 1000 μM cadmium chloride, respectively. As a result, the GE *P. aeruginosa* has shown great adsorption capacity than WT *P. aeruginosa*. In 50 μM Cd(II) broth, the GE *P. aeruginosa* with the surface-displayed CadR was able to adsorb Cd(II) with a capacity of approximately 16.4 μmol/g cell, which are 7-fold higher than the WT *P. aeruginosa*. In 100 μM Cd(II) broth, the GE *P. aeruginosa* with the surface-displayed CadR was able to adsorb Cd(II) with a capacity of approximately 32.7 μmol/g cell. In 200 μM Cd (II) broth, the GE *P. aeruginosa* with the surface-displayed CadR was able to adsorb cadmium ions with a capacity of approximately 65.7 μmol/g cell. In 500 μM Cd(II) broth, the GE *P. aeruginosa* with the surface-displayed CadR was able to adsorb Cd(II) with a capacity of approximately 128.0 μmol/g cell. In 1000 μM broth, the GE *P. aeruginosa* with the surface-displayed CadR was able to adsorb Cd(II) with a capacity of approximately 131.2 μmol/g cell (Fig. 3a). The sorption isotherm of Cd(II) by the GE *P. aeruginosa* bacteria represents the equilibrium distribution of Cd(II) between bacteria and aqueous phase. Cd(II) uptake by GE *P. aeruginosa* bacteria increased with the rising of initial Cd(II) concentration (Fig. 4) and the absorption data were fit to linear form of the Langmuir isotherm model:

$$\frac{C_f}{q} = \frac{C_f}{q_{\max}} + \frac{1}{bq_{\max}} \quad (1)$$

where q is the metal uptake and $q_{\max}$ is the maximum adsorption capacity; C_f is the final Cd(II) concentration at equilibrium; b is the Langmuir constant, related to the adsorption energy. Our experimental data fit the Langmuir model with good linear correlation, suggesting that the adsorption of Cd(II) by the GE *P. aeruginosa* bacteria followed a physicochemical, equilibrated and saturatable mechanism. The maximum Cd(II) adsorption capacity was calculated

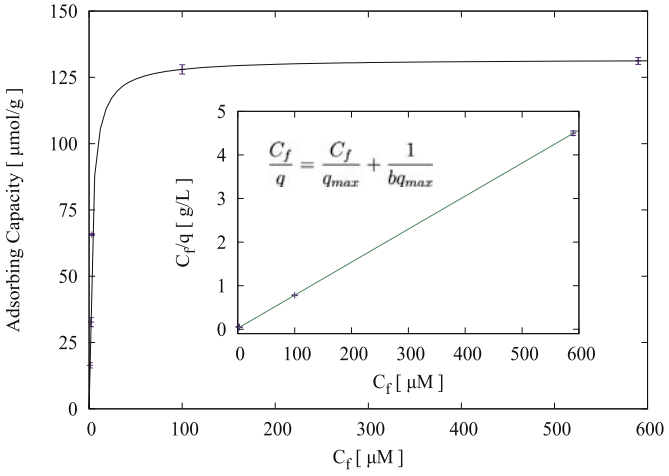

Fig. 4. Sorption isotherm of Cd(II) by GE *P. aeruginosa* bacteria. Insert: Langmuir transformation for the sorption isotherm of Cd(II).

about 131.9 μmol/g cells by using the Langmuir equation. It also confirms that the protein is expressed on the surface of the bacteria from another aspect.

GE *P. aeruginosa* with the surface-displayed CadR has exhibited excellent adsorption capacity, but WT *P. aeruginosa* cannot grow in high concentration Cd(II) broth. Besides, the rates of effective adsorption of CadR displayed cells in the Cd(II) concentration of 50 μM, 100 μM, 200 μM, 500 μM or 1000 μM ranged from 41% to 98.5%, however, the WT *P. aeruginosa* cells had a low cadmium ion adsorption capacity (about 2.4 μmol/g cells) at concentrations because of the negative charge gathering on the surface of WT *P. aeruginosa* lower than 50 μM and cannot grow in higher Cd(II) concentrations. In conclusion, the CadR displaying on the *P. aeruginosa* surface has significantly enhanced Cd(II) adsorption capacity of *P. aeruginosa*. The GE *P. aeruginosa* could even survive and grow without visible defect at the Cd(II) concentration of 1000 μM. A high Cd(II) adsorption capacity is crucial to practical application for Cd(II) bioremediation from actual environment.

3.4. Responses to other divalent metal stress

The lack of metal selectivity greatly affects the adsorption efficiency (Wei et al., 2014). Specific adsorption of heavy metals has attracted significant attention. The bacterial outer cell membrane has a complex chemical structure; therefore, nonspecific adsorption of other metal ions must occur to interfere with Cd(II) selective adsorption (Wei et al., 2014). Their growth in cadmium solution is quite different, while, under other divalent metal stress (Mn(II), Cu(II), Zn(II), Hg(II) and Pb(II)), the behavior of GE *P. aeruginosa* was very similar to that of the wildtype bacteria (E-supplement). In agreement with previous studies (Liu et al., 2015), the *cadR* transgenic bacteria have higher selectivity towards Cd(II) over Mn(II), Cu(II), Zn(II), Hg(II) and Pb(II), and the WT *P. aeruginosa* has a high tolerance to Mn(II). Besides, the coexistence of low concentration divalent ions has no significant effect on the adsorption capacity, suggesting that it has the potential to be used in a single cadmium polluted water (E-supplement).

3.5. Effect of pH and temperature on adsorption capacity

There are many factors that affect the adsorption capacity of GE *P. aeruginosa* to Cd(II) in aqueous environment. We discussed the two most common environmental factors, pH and temperature. The adsorption experiments of 200 μM, 500 μM or 1000 μM Cd(II) by the GE *P. aeruginosa* were performed in the LB broth under different pH. As shown in Fig. 5a, the Cd(II) binding capacity of the surface-displayed bacteria was the highest at pH=7.0 and decreased gradually to 2.6 μmol/g cells with pH decreasing to 3.0. The nitrogen atom of amine group is able to bind to a proton or a metal ion by sharing the electron pair (Yin et al., 2016). Under neutral environment, the electrical attraction of the Cd(II) with the lone pairs of nitrogen atom is stronger than that of hydrogen ion, resulting in the binding of Cd(II) to nitrogen. At low pH values, on the one hand, the binding Cd(II) can be replaced by the hydrogen ion because of its high concentration. On the other hand, the acidic environment inhibits the activity of enzymes in cells and leads to a decrease in cellular activity.

When GE *P. aeruginosa* cultivated in gradient temperature, GE *P. aeruginosa* has showed optimum adsorption capacity at 30 °C. When the temperature is below 30 °C, the adsorption capacity increases with the increase of temperature, however, when the temperature is over 30 °C, the adsorption capacity decreases with the increase of temperature. As shown in Fig. 5b, the adsorption capacity in 30 °C was 2-fold to 15 °C, and the adsorption capacity drops sharply below 10 °C. We speculate that enzyme of *P. aeruginosa* has optimal activity at 30 °C, so it has the highest physiological activity at 30 °C.

4. Conclusions

Currently, biological treatment is highly concerned because of its environmental-friendly and low cost. Inspired by the Cd(II)-specific regulation CadR protein, we have developed a chromosomal engineering surface display system on the *P. aeruginosa*. The results have verified that CadR anchoring on the outer membrane by INP is remarkably enhanced the selective adsorption of Cd(II) after the surface display on the *P. aeruginosa*. In the meanwhile, the excellent stability of chromosomal gene expression makes it promising for the application of heavy metal treatment. Therefore, the developed surface display system can be utilized as a simple method for the simultaneous remediation of Cd(II).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.04.229.

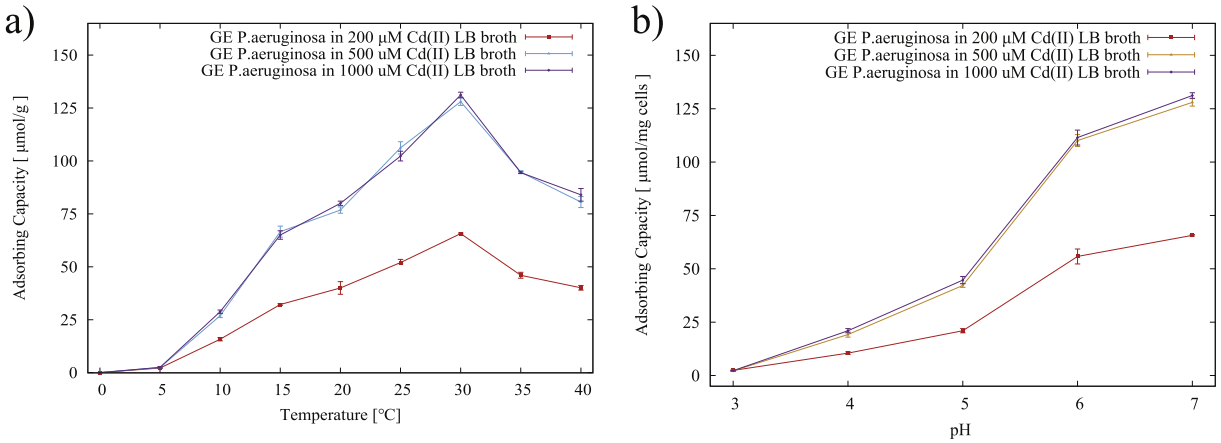

Fig. 5. a) Cd(II) adsorption capacity of GE *P. aeruginosa* in different pH. b) Cd(II) adsorption capacity of GE *P. aeruginosa* in different temperature.

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