Science of the Total Environment
Manuscript Draft

Manuscript Number: STOTEN-D-18-01401R1

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

Article Type: Research Paper

Keywords: CadR, Chromosomal surface display, Pseudomonas aeruginosa, Cadmium, Bioremediation

Abstract: Genetically engineered bacteria for pollution control of heavy metal have been widely studied, however, using Pseudomonas aeruginos (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 was still flourished in the 30th generation in the LB broth which contained 100 μM Cd(II), exhibiting a excellent genetic stability. Chromosomal expressed P.aeruginosa showed a 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.

Response to Reviewers: Dear editor,
We quite appreciate the valuable comments of editor and reviewers on our manuscript of 'STOTEN-D-18-01401'. The suggestions are very helpful for us and we have incorporated them into the revised paper. We checked the manuscript carefully, and revised it according to the comments of editor and reviewers. We are sure that points listed in the checklist are addressed. Specific responses to the comments were provided one by one. All the work is to improve the quality and the understanding of our paper.
On behalf of my co-authors, I would like to provide a detailed list (point by point)
of responses to each item of the comments. We also highlighted our revisions in the
manuscript in red so that editor/reviewers could easily identify them. We hope that the
editor and the reviewers will be satisfied with our responses to the comments and the
revisions for our original manuscript.
Thanks and best regards.
Yours sincerely,
Guangming Zeng
E-mail address: zgming@hnu.edu.cn

Revisions based on Reviewer #1: Manuscript "Chromosomal expression of CadR on Pseudomonas aeruginosa for the removal of Cd(II) from aqueous solutions" constructed a genetically engineered bacterium, Pseudomonas aeruginosa, which emerged CdR gene into chromosomal, expressed CadR in its membrane, and can be potentially used to remove Cd(II) from aqueous solutions. Basically, the manuscript was well organized and of reference value. I would suggest publication after a minor revision.

Comment 1. As author has mentioned in "Introduction" part, P. puida has CadR gene and is also used to control heavy metal pollution. Why not choose P. puida?

Response：

We are much grateful for your valuable suggestion. On the one hand, Jian Chen et al. demonstrated that a strain of Pseudomonas putida KT2440 endowed with chromosomal expression of the arsM gene encoding the As(III) S-adenosylmethionine (SAM) methyltransfase from Rhodopseudomonas palustris to remove arsenic from contaminated soil. The arsenic(III) S-adenosylmethionine methyltransferase (arsM) gene were able to sequentially methylate toxic inorganic arsenic to less toxic pentavalent methylated arsenicals such as methylarsenate (MAs(V)), dimethylarsenate (DMAs(V)) and trimethylarsine oxide (TMAs(V)O). Moreover, the arsM is a gene of arsenic specific response. Therefore, we find that the expression of another heavy metal response protein in Pseudomonas putinosa is not significant. On the other hand, he heavy metal binding protein has not been used in Pseudomonas aeruginosa, This is a blank space for research, so we chose Pseudomonas aeruginosa as a host bacterium to understand the feasibility of Pseudomonas aeruginosa as a host for heavy metal remediation.

Comment 2. In Page 13 line 207, didnt should be corrected to didn't.

Response:

We really appreciate your kind suggestion. We corrected the spelling of the word, revision of detail can be found in the revised manuscript in line 207, page 13.

Conmment 3. In Page 15 line 235 "to chromosomal express CadR" should be corrected to "To achieve CadR chromosomal expression".

Response:

Many thanks for your useful comment. We modified the expression of the sentence according to your opinion, revision of detail can be found in the revised manuscript in line 236, page 15.

Revisions based on Reviewer #2: In this study, heavy metal-binding proteins CadR was first expressed on the surface of Pseudomonas aeruginosa cell membranes, and the engineered bacteria exhibited excellent cadmium ion adsorption capability, and exhibited strong genetic stability due to chromosomal expression. This provides a direction for the expression of foreign genes, especially heavy metal-binding protein genes, in Pseudomonas aeruginosa. The study has important results may be useful to science. The manuscript is well written and organized. Overall I recommend this manuscript for publication after minor revision.

Comment 1. Page 9, Figure 1 is hard to read, maybe you can convert it to a linear map to make it easier to read.

Response:

Thank you for the valuable comment. We have changed the plasmid map to a linear map in your opinion, and now it is easier to read than before. Revision of detail can be found in the revised manuscript in page 10.

Comment 2. Page 19, line 278-285, Why do you use a single clone for genetic stability experiments, instead of using 100 clones to test the loss probability of their cadmium ion resistance?

Response:

We really appreciate your kind suggestion. We have learned from the previous literature, the chromosomal expression is more stable than plasmid expression, so we only choose single monoclonal to study the stability of breeding instead of using 100 monoclonal to investigate whether chromosome expression is more stable.

Comment 3. The paper is written correctly. I only find three errata:

1. Page 6, line 46: "palasmid" should be "plasmid".
2. Page 14, line 216: "tempreture" should be "temperature".
3. Page 19, line 286: "Incontrast" should be "In contrast"

Response:

We are much grateful for your valuable suggestion. We corrected the spelling of the words, revision of detail can be found in the revised manuscript in line 46, page 4; line 216, page 14 and line 287, page 16.

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

Xiang Tang[a,b], Guangming Zeng[a,b,*], Changzheng Fan[a,b,**], Man Zhou[a,b], Lin Tang[a,b], Jingjing Zhu[a,b], Jia Wan[a,b], Danlian Huang[a,b], Ming Chen[a,b], Piao Xu[a,b], Chen Zhang[a,b], Weiping Xiong[a,b]

[a]*College of Environmental Science and Engineering, Hunan University, Changsha 410082, China*
[b]*Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, China*

*corresponding author. phone: +86-731-8-882370, fax: +86-731-8-8823701.
**corresponding author. phone: +86-13787102427.
Email addresses: fancz@hnu.edu.cn (Guangming Zeng), zgming@hnu.edu.cn (Changzheng Fan)

*Author agreement

Author's agreement

We the undersigned declare that the manuscript entitled "**Chromosomal expression of CadR on Pseudomonas aeruginosa for the removal of Cd(II) from aqueous solutions** " is original, has not been full or partly published before, and is not currently being considered for publication elsewhere.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by the undersigned.

We understand that the Corresponding Author is the sole contact for the editorial process. The corresponding author "**Chromosomal expression of CadR on Pseudomonas aeruginosa for the removal of Cd(II) from aqueous solutions** " is responsible for communicating with the other authors about process, submissions of revisions, and final approval of proofs."

Signature of all authors :

Dear editor,

We quite appreciate the valuable comments of editor and reviewers on our manuscript of 'STOTEN-D-18-01401'. The suggestions are very helpful for us and we have incorporated them into the revised paper. We checked the manuscript carefully, and revised it according to the comments of editor and reviewers. We are sure that points listed in the checklist are addressed. Specific responses to the comments were provided one by one. All the work is to improve the quality and the understanding of our paper.

On behalf of my co-authors, I would like to provide a detailed list (point by point) of responses to each item of the comments. We also highlighted our revisions in the manuscript in red so that editor/reviewers could easily identify them. We hope that the editor and the reviewers will be satisfied with our responses to the comments and the revisions for our original manuscript.

Thanks and best regards.

Yours sincerely,

Guangming Zeng

E-mail address: zgming@hnu.edu.cn

Revisions based on Reviewer #1: Manuscript "Chromosomal expression of CadR on *Pseudomonas aeruginosa* for the removal of Cd(II) from aqueous solutions" constructed a genetically engineered bacterium, *Pseudomonas aeruginosa*, which emerged CdR gene into chromosomal, expressed CadR in its membrane, and can be potentially used to remove Cd(II) from aqueous solutions. Basically, the manuscript was well organized and of reference value. I would suggest publication after a minor revision.

Comment 1. As author has mentioned in "Introduction" part, *P. puida* has CadR gene and is also used to control heavy metal pollution. Why not choose *P. puida*?

Response:

We are much grateful for your valuable suggestion. On the one hand, Jian Chen et al. demonstrated that a strain of *Pseudomonas putida KT2440* endowed with chromosomal expression of the *arsM* gene encoding the As(III) S-adenosylmethionine (SAM) methyltransfase from *Rhodopseudomonas palustris* to remove arsenic from contaminated soil. The arsenic(III) S-adenosylmethionine methyltransferase (*arsM*) gene were able to sequentially methylate toxic inorganic arsenic to less toxic pentavalent methylated arsenicals such as methylarsenate (MAs(V)), dimethylarsenate (DMAs(V)) and trimethylarsine oxide (TMAs(V)O). Moreover, the *arsM* is a gene of arsenic specific response. Therefore, we find that the expression of another heavy metal response protein in *Pseudomonas stinosa* is not significant. On the other hand, he heavy metal binding protein has not been used in *Pseudomonas aeruginosa*, This is a blank space for research, so we chose *Pseudomonas aeruginosa* as a host bacterium

to understand the feasibility of *Pseudomonas aeruginosa* as a host for heavy metal remediation.

Comment 2. In Page 13 line 207, didnt should be corrected to didn't.

Response:

We really appreciate your kind suggestion. We corrected the spelling of the word, revision of detail can be found in the revised manuscript in line 207, page 13.

Conmment 3. In Page 15 line 235, "to chromosomal express CadR" should be corrected to "To achieve CadR chromosomal expression".

Response:

Many thanks for your useful comment. We modified the expression of the sentence according to your opinion, revision of detail can be found in the revised manuscript in line 236, page 15.

Revisions based on Reviewer #2: In this study, heavy metal-binding proteins CadR was first expressed on the surface of *Pseudomonas aeruginosa* cell membranes, and the engineered bacteria exhibited excellent cadmium ion adsorption capability, and exhibited strong genetic stability due to chromosomal expression. This provides a direction for the expression of foreign genes, especially heavy metal-binding protein genes, in *Pseudomonas aeruginosa*. The study has important results may be useful to science. The manuscript is well written and organized. Overall I recommend this manuscript for publication after minor revision.

Comment 1. Page 9, Figure 1 is hard to read, maybe you can convert it to a linear

map to make it easier to read.

Response:

Thank you for the valuable comment. We have changed the plasmid map to a linear map in your opinion, and now it is easier to read than before. Revision of detail can be found in the revised manuscript in page 10.

Comment 2. Page 19, line 278-285, Why do you use a single clone for genetic stability experiments, instead of using 100 clones to test the loss probability of their cadmium ion resistance?

Response:

We really appreciate your kind suggestion. We have learned from the previous literature, the chromosomal expression is more stable than plasmid expression, so we only choose single monoclonal to study the stability of breeding instead of using 100 monoclonal to investigate whether chromosome expression is more stable.

Comment 3. The paper is written correctly. I only find three errata:

1. Page 6, line 46: "palasmid" should be "plasmid".

2. Page 14, line 216: "tempreture" should be "temperature".

3. Page 19, line 286: "Incontrast" should be "In contrast"

Response:

We are much grateful for your valuable suggestion. We corrected the spelling of the words, revision of detail can be found in the revised manuscript in line 46, page 4; line 216, page 14 and line 287, page 16.

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

Xiang Tang[a,b], Guangming Zeng[a,b,*], Changzheng Fan[a,b,**], Man Zhou[a,b], Lin Tang[a,b], Jingjing Zhu[a,b], Jia Wan[a,b], Danlian Huang[a,b], Ming Chen[a,b], Piao Xu[a,b], Chen Zhang[a,b], Weiping Xiong[a,b]

[a]*College of Environmental Science and Engineering, Hunan University, Changsha 410082, China*
[b]*Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, China*

Abstract

Genetically engineered bacteria for pollution control of heavy metal have been widely studied, however, using *Pseudomonas aeruginos* (*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* was still flourished in the 30th generation in the LB broth which contained 100 μM Cd(II), exhibiting a excellent genetic stability. Chromosomal expressed *P.aeruginosa* showed a adsorption capacity of

*corresponding author. phone: +86-731-8-882370, fax: +86-731-8-8823701.
**corresponding author. phone: +86-13787102427.
Email addresses: zgming@hnu.edu.cn (Guangming Zeng), fancz@hnu.edu.cn (Changzheng Fan)

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.
Keywords: CadR, Chromosomal surface display, *Pseudomonas aeruginosa*, Cadmium, Bioremediation

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 bring 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 humans

health. Therefore, it is an urgent demand to seek for an efficient and environmentally compatible method to remove or detoxify heavy metals(Xu et al., 2012b; Chen et al., 2016; Song et al., 2017). Currently, the pollution treatment technologies contains 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 provides 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 ad-

sorption), 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 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 have 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 include plasmid expression and chromosome expression, which 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 applica-

tions(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 are comprised of 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 (1200-1500aa) and neither the C-terminal domain nor the central repeating domain (CRD) of the INPs harbours signal peptide sequences(Li et al., 2012). Therefore, the INP-N (the N-terminal domain) or the INP-NC (containing only the N-terminaland 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 the chromosome of *P.aeruginosa* by tripartite mating. This is the first use of *P.eruginosa* 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 gene 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 was synthetized by GENEWIZ. The *INP* and *cadR* were conjugated through touchdown-PCR: at first, Primer 1 (5'-AAGCTCTAGAGCATGAA CCGCGAGAAGGTGCTGGCCCT-3' (XbaI site underlined)) and Primer 2 (5'-GCCAGCTCGCCGATCTTCATGAACTCGATCTCGCGGGTG-3') was employed to amplify the INP coding gene; Primer 3 (5'-CACCCGCGAGATCGAGTTCATGA AGATCGGCGAGCTGGC-3') and Primer 4 (5'-AATATCGGGATCCCGGTGGCC GTGGCTGCGG CCCACGT-3' (BamHI site underlined)) was employed to amplify the *cadR* coding gene. In overlap PCR process, Primer 1 and Primer 4 was utilized to amplify the fused INP and *cadR* gene. The overlap PCR profile was as follows: 95 °C for 5 minutes; 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 minutes. 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)) was 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 was 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.

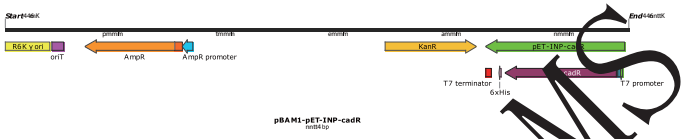


Figure 1: Schematic representation of the main features of the version of mini-Tn5 engineered in the pBAM1 backbone, containing the CadR coding gene fused with the INP encoding gene, and the *INP-cadR* insert to T7 promoter.

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 bacterias 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 hours 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 contains 100 μM cadmium and 50 μg /L kanamycin) in appropriate diluted concentration, then cultured in selective medium at 30 °C for 36 hours. 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 that containing 500 μM Cd(II) and 50 μg/mL of kanamycin, and the cultures was grown to an OD_{600} = 0.5-0.7 after 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 that containing 5% dry milk and allowed to incubated with anti-His Mouse monoclonal Antibody (CMCTAG) diluted in 1:2000 at room temperature for two hours. After washing three times with TBST (10 min for once), the blot was incubated with peroxidase-conjugated goat antimouse IgG (H+L)-HRP (CMCTAG) at 1:5000 dilution in blocking buffer for one hour. 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 hours by gentle shaking, and then, 10 L of the cultures was added to 1.0 mL fresh selective LB broth for 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 hours 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 five minutes at room temperature,and resuspended in 1 mL PBS buffer and 10 μL cultures before incubated to LB broth that 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 hours with 120 rpm shaking. After incubation, the cells were separated from the reaction solution by centrifugation at 5000 rpm for ten minutes 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 (AAS). 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 that 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 hours. 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 hours. 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 over 7.0, because $Cd(OH)_2$ is the dominant species in solution. The GE *P.aeruginosa* was inoculated to selective medium (contained 100

209 μM Cd(II) and 50 μg/mL kanamycin) and cultured at 30 °C overnight, and then
210 pick a single colony for amplification in selective broth (contained 100 μM Cd(II)
211 and 50 g/mL kanamycin). 10 μL cultures were inoculated to 20 mL LB broth that
212 contain 200 μM, 500 μM or 1000 μM Cd(II), all concentrations are matching with
213 different pH. The inoculated culture was cultured at 30 °C with gentle shaking
214 for 12 hours. After centrifugation (12,000 rpm, 5 min), the supernatant was col-
215 lected for analyzing the concentrations of Cd(II) by AFS. To investigate the effect
216 of temperature to Cd(II) adsorption capacity of the proposed GE *P.aeruginosa*,
217 we detected its adsorption capacity for different Cd(II) concentration (200 μM,
218 500 μM or 1000 μM) solutions at different temperatures(0 °C-40 °C). Firstly, GE
219 *P.aeruginosa* was inoculated on the selection LB plate at 30 °C for 12 hours. Af-
220 terwards, monoclonal colony were inoculated to Cd(II)-contained LB broth and
221 incubated at different temperature with for overnight. The detection method is
222 consistent with the above.

223 **3. Results and Discussion**

224 *3.1. Chromosomal Expression of CadR*

225 Metallothionein (MT) and plant chelating peptides have been widely used for
226 microbiological surface display. They are all rich in cysteine and are not spe-

cific to the absorption of heavy metals. When there are several kinds of metal ions in the environment of existence, competitive target of heavy metal ions of non target metal ions will be in metal binding sites, the target of heavy metal ions for binding site affinity than the target metal ions will occupy the preferred 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 presence of 981 bp fragment has initial verified that overlap PCR had undergone as expected (E-supplement). The pBAM1 have the mini-Tn5 transposon that can randomly inserted 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 initial verified by primer T7-F and primer T7-R, original T7 promoter gene was 380 bp, the recon-

structed 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 displaying 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) was close to their theoretical values.

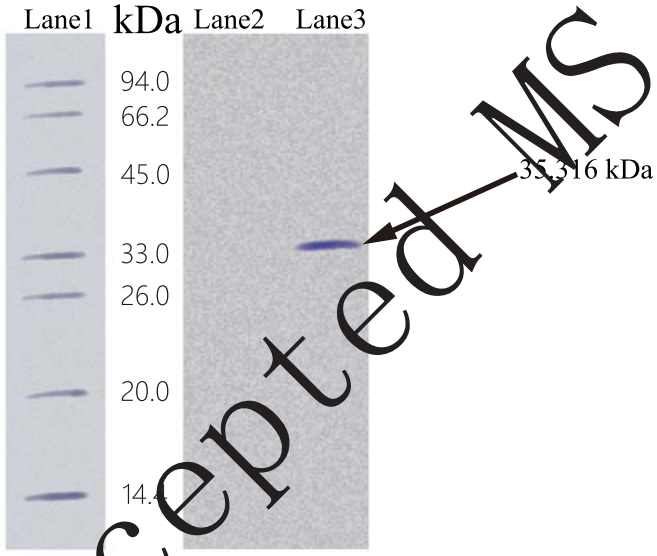


Figure 2: Western blot analysis of CadR. Lane 1, Protein standards. Lane 2, WT *P. aeruginosa*. Lane 3, GE *P. aeruginosa*.

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* show high tolerance to Cd(II) than *E.coli BL21(DE3)* that 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-replication in *E.coli BL21(DE3)* without the λpir protein(Martłnez-Garcła 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 were essential for their application in natural polluted environment. To examine whether chromosomal expression have 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 have shown the OD_{600} of selective LB broth after 12 hours gentle shaking at 30 °C in the Pass-generation assay. In *Con E.coli* group, the OD_{600} was consistently de-

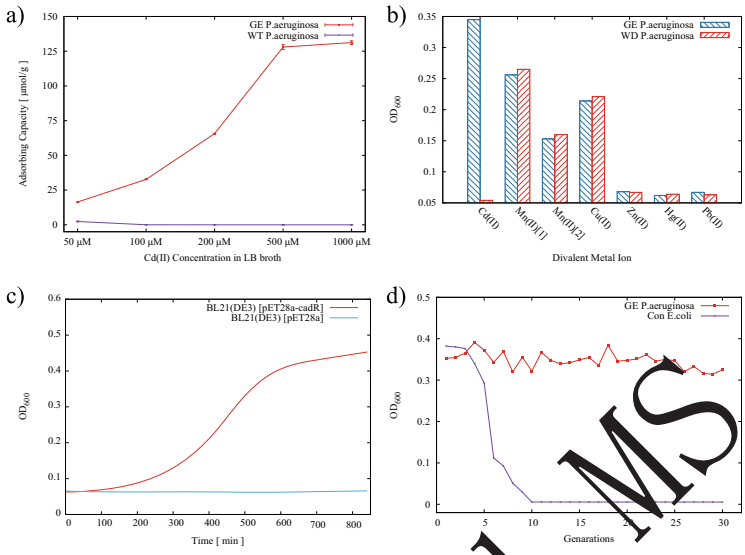


Figure 3: a) The plasmid pET-*cadR* and plasmid pET28a was transformed to *E.coli BL21(DE3)* compent cells, and its growth curve in LB broth (500 μM/L) was tested. b) The OD600 of GE *P. aeruginosa* when inoculated to LB broth under different divalent metal stress after 8 hours gentle shaking. c) Cd(II) 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 hours gentle shaking in 30°C in the Pass-generation assay.

creasing 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 *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 were 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 were 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 were 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 were 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 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 was 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 that gathering on the surface of WT *P.aeruginosa* that low than 50

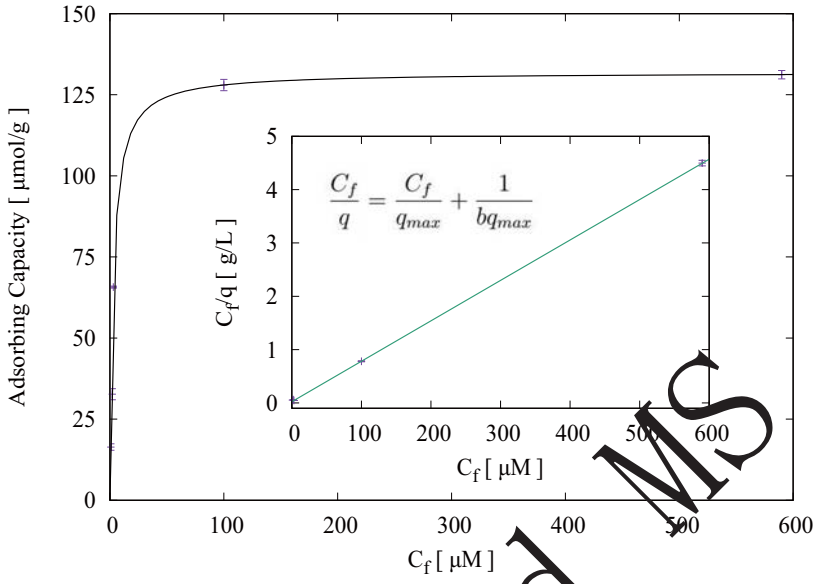


Figure 4: Sorption isotherm of Cd(II) by GE *P.aeruginosa* bacteria. Insert: Langmuir transformation for the sorption isotherm of Cd(II).

μM and cannot grown 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, non-specific 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 high selectivity towards Cd(II) over Mn(II), Cu(II), Zn(II), Hg(II) and Pb(II), and the WT *P.aeruginosa* have 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 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, acid environment was inhibit the cell activity.

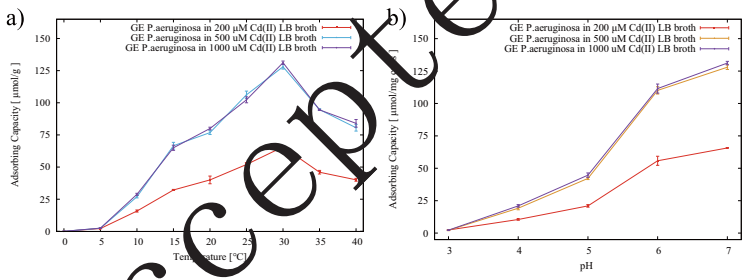


Figure 5: a) Cd(II) adsorption capacity of GE *P. aeruginosa* in different pH. b) Cd(II) adsorption capacity of GE *P. aeruginosa* in different temperature.

When GE *P.aeruginosa* cultivated in gradient temperature, GE *P.aeruginosa* have 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* have 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).

acknowledgement

The study was fnancially supported by the National Natural Science Foundation of China (51521006, 51679084, 51378190, 51579096, 51508177, 51709101)

385 and the Program for Changjiang Scholars and Innovative Research Team in Uni-
386 versity (IRT-13R17).

387 Baldwin, D. R., Marshall, W. J., 1999. Heavy metal poisoning and its laboratory
388 investigation. Annals of Clinical Biochemistry 36 (3), 267–300.

389 Brocklehurst, K., Megit, S., Morby, A., 2003. Characterisation of cadr from pseu-
390 domonas aeruginosa: a cd (ii)-responsive merr homologue. Biochemical and
391 biophysical research communications 308 (2), 234–239.

392 Brown, N. L., Stoyanov, J. V., Kidd, S. P., Hobman, J. L., 2003. The merr family
393 of transcriptional regulators. FEMS microbiology reviews 27 (2-3), 145–163.

394 Chen, J., Sun, G.-X., Wang, X.-Y., Lorenzo, V. d., Rosen, B. P., Zhu, Y.-G., 2014.
395 Volatilization of arsenic from polluted soil by pseudomonas putida engineered
396 for expression of the arsm arsenic (iii) s-adenosine methyltransferase gene. En-
397 vironmental science & technology 48 (17), 10337–10344.

398 Chen, M., Xu, P., Zeng, G., Yang, C., Huang, D., Zhang, J., 2015. Bioremediation
399 of soils contaminated with polycyclic aromatic hydrocarbons, petroleum,
400 pesticides, chlorophenols and heavy metals by composting: Applications,
401 microbes and future research needs. Biotechnology Advances 33 (6, Part 1),

745 – 755.

URL http://www.sciencedirect.com/science/article/pii/S0734975015300021

Chen, Z., Cui, L., Zeng, G., Huang, D., Lin, T., Yang, C., Zhou, Y., Lei, Q., Min, C., 2016. Nanoporous au-based chronocoulometric aptasensor for amplified detection of pb 2+ using dnazyme modified with au nanoparticles. Biosensors & Bioelectronics 81, 61.

Cheng, Y., He, H., Yang, C., Zeng, G., Li, X., Chen, H., Yu, G., 2016. Challenges and solutions for biofiltration of hydrophobic volatile organic compounds. Biotechnology Advances 34 (6), 1091.

Chiu, T.-Y., Chen, P.-H., Chang, C.-L., Yang, D.-M., 2013. Live-cell dynamic sensing of cd2+ with a fret-based indicator. PloS one 8 (6), e65853.

Chungjatupornchai, W., Fa-aroonsawat, S., 2009. Translocation of green fluorescent protein to cyanobacterial periplasm using ice nucleation protein. The Journal of Microbiology 47 (2), 187–192.

Cui, J., Liu, T., Li, F., Yi, J., Liu, C., Yu, H., 2017. Silica nanoparticles alleviate cadmium toxicity in rice cells: Mechanisms and size effects. Environmental Pollution 228 (Supplement C), 363 – 369.

URL http://www.sciencedirect.com/science/article/pii/S0269749116318486

420 Fan, L.-H., Liu, N., Yu, M.-R., Yang, S.-T., Chen, H.-L., 2011. Cell surface dis-
421 play of carbonic anhydrase on escherichia coli using ice nucleation protein for
422 co2 sequestration. Biotechnology and bioengineering 108 (12), 2853–2864.

423 Gong, J. L., Wang, B., Zeng, G. M., Yang, C. P., Niu, C. G., Niu, Q. Y., Zhou,
424 W. J., Yi, L., 2009. Removal of cationic dyes from aqueous solution using mag-
425 netic multi-wall carbon nanotube nanocomposite as adsorbent. Journal of Haz-
426 ardous Materials 164 (2C3), 1517–1522.

427 Huang, C. C., Hsieh, J. L., Su, C. C., Tseng, C. F., Lin, P. J., Chang, J. S., 2003.
428 Polypeptides for heavy-metal biosorption:capacity and specificity of two het-
429 erogeneous merp proteins. Enzyme & Microbial Technology 33 (4), 379–385.

430 Khodi, S., Latifi, A. M., Saadati, M., Mirzaei, M., Aghamollaei, H., 2012. Surface
431 display of organophosphorus hydrolase on e. coli using n-terminal domain of
432 ice nucleation protein inav. Journal of Microbiology & Biotechnology 22 (2),
433 234.

434 Kozloff, L., Turner, M., Arellano, F., 1991. Formation of bacterial membrane ice-
435 nucleating lipoglycoprotein complexes. Journal of bacteriology 173 (20), 6528–
436 6536.

437 Lee, S.-W., Glickmann, E., Cooksey, D. A., 2001. Chromosomal locus for cad-

mium resistance in pseudomonas putida consisting of a cadmium-transporting atpase and a merr family response regulator. Applied and environmental microbiology 67 (4), 1437–1444.

Li, Q., Yan, Q., Chen, J., He, Y., Wang, J., Zhang, H., Yu, Z., Li, L., 2012. Molecular characterization of an ice nucleation protein variant (inaq) from pseudomonas syringae and the analysis of its transmembrane transport activity in escherichia coli. International journal of biological sciences 8 (8), 1097.

Li, Q., Yu, Z., Shao, X., He, J., Li, L., 2009. Improved phosphate biosorption by bacterial surface display of phosphate-binding protein utilizing ice nucleation protein. FEMS microbiology letters 299 (1), 44–52.

Liu, Q., Yuan, F., Liang, Y., Li, Z., 2015. Cadmium adsorption by e. coli with surface displayed cadr. RSC Advances 5 (21), 16089–16092.

Long, F., Gong, J. L., Zeng, G. M., Chen, L., Wang, X. Y., Deng, J. H., Niu, Q. Y., Zhang, H. Y., Zhang, X. R., 2011. Removal of phosphate from aqueous solution by magnetic feczr binary oxide. Chemical Engineering Journal 171 (2), 448–455.

Malik, A., 2004. Metal bioremediation through growing cells. Environment International 30 (2), 261–78.

Martłnez-Garcła, E., Calles, B., Arvalo-Rodrłguez, M., de Lorenzo, V., 2011. pbam1: an all-synthetic genetic tool for analysis and construction of complex bacterial phenotypes. BMC Microbiology 11 (1), 38.

Ming, C., Qin, X., Zeng, G., Jian, L., 2016. Impacts of human activity modes and climate on heavy metal "spread" in groundwater are biased. Chemosphere 152, 439–445.

Ren, X., Zeng, G., Tang, L., Wang, J., Wan, J., Liu, Y., Yu, J., Yi, H., Ye, S., Deng, R., 2017. Sorption, transport and biodegradation - an insight into bioavailability of persistent organic pollutants in soil. Science of the Total Environment 610-611, 1154–1163.

Schmid, D., Pridmore, D., Capitani, G., Battistutta, R., Neeser, J.-R., Jann, A., 1997. Molecular organization of the ice nucleation protein inav from pseudomonas syringae. FEBS letters 414 (3), 590–594.

Smith, G. P., 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228, 1315–1318.

Song, B., Zeng, G., Gong, J., Jie, L., Xu, P., Liu, Z., Yi, Z., Chen, Z., Min, C., Yang, L., 2017. Evaluation methods for assessing effectiveness of in situ reme-

diation of soil and sediment contaminated with organic pollutants and heavy metals. Environment International 105, 43.

Tan, X., Liu, Y., Zeng, G., Wang, X., Hu, X., Gu, Y., Yang, Z., 2015. Application of biochar for the removal of pollutants from aqueous solutions. Chemosphere 125 (Supplement C), 70 – 85.
URL http://www.sciencedirect.com/science/article/pii/S0045653514015008

Tang, W. W., Zeng, G. M., Gong, J. L., Liang, J., Xu, P., Zhang, C., Huang, B. B., 2014. Impact of humic/fulvic acid on the removal of heavy metals from aqueous solutions using nanomaterials: a review. Science of the Total Environment 468-469, 1014.

Tao, H.-C., Peng, Z.-W., Li, P.-S., Yu, T.-A., Su, J., 2013. Optimizing cadmium and mercury specificity of cadr-based e. coli biosensors by redesign of cadr. Biotechnology letters 35 (8), 1253–1258.

Wei, W., Liu, X., Sun, P., Wang, X., Zhu, H., Hong, M., Mao, Z. W., Zhao, J., 2014. Simple whole-cell biodetection and bioremediation of heavy metals based on an engineered lead-specific operon. Environmental Science & Technology 48 (6), 3363–71.

Wolber, P. K., Deininger, C. A., Southworth, M. W., Vandekerckhove, J.,

Van Montagu, M., Warren, G. J., 1986. Identification and purification of a bacterial ice-nucleation protein. Proceedings of the National Academy of Sciences 83 (19), 7256–7260.

Wu, C. H., Le, D., Mulchandani, A., Chen, W., 2009. Optimization of a whole-cell cadmium sensor with a toggle gene circuit. Biotechnology progress 25 (3), 898–903.

Wu, H., Lai, C., Zeng, G., Liang, J., Chen, J., Xu, J., Dai, J., Li, X., Liu, J., Chen, M., 2017. The interactions of composting and biochar and their implications for soil amendment and pollution remediation: a review. Critical Reviews in Biotechnology 37 (6), 754.

Xu, P., Zeng, G. M., Huang, D. L., Feng, C. L., Hu, S., Zhao, M. H., Lai, C., Wei, Z., Huang, C., Xie, G. X., Liu, Z. F., 2012a. Use of iron oxide nanomaterials in wastewater treatment: A review. Science of The Total Environment 424 (Supplement C), 1 – 10.
URL http://www.sciencedirect.com/science/article/pii/S0048969712001982

Xu, P., Zeng, G. M., Huang, D. L., Lai, C., Zhao, M. H., Wei, Z., Li, N. J., Huang, C., Xie, G. X., 2012b. Adsorption of pb(ii) by iron oxide nanoparticles immobi-

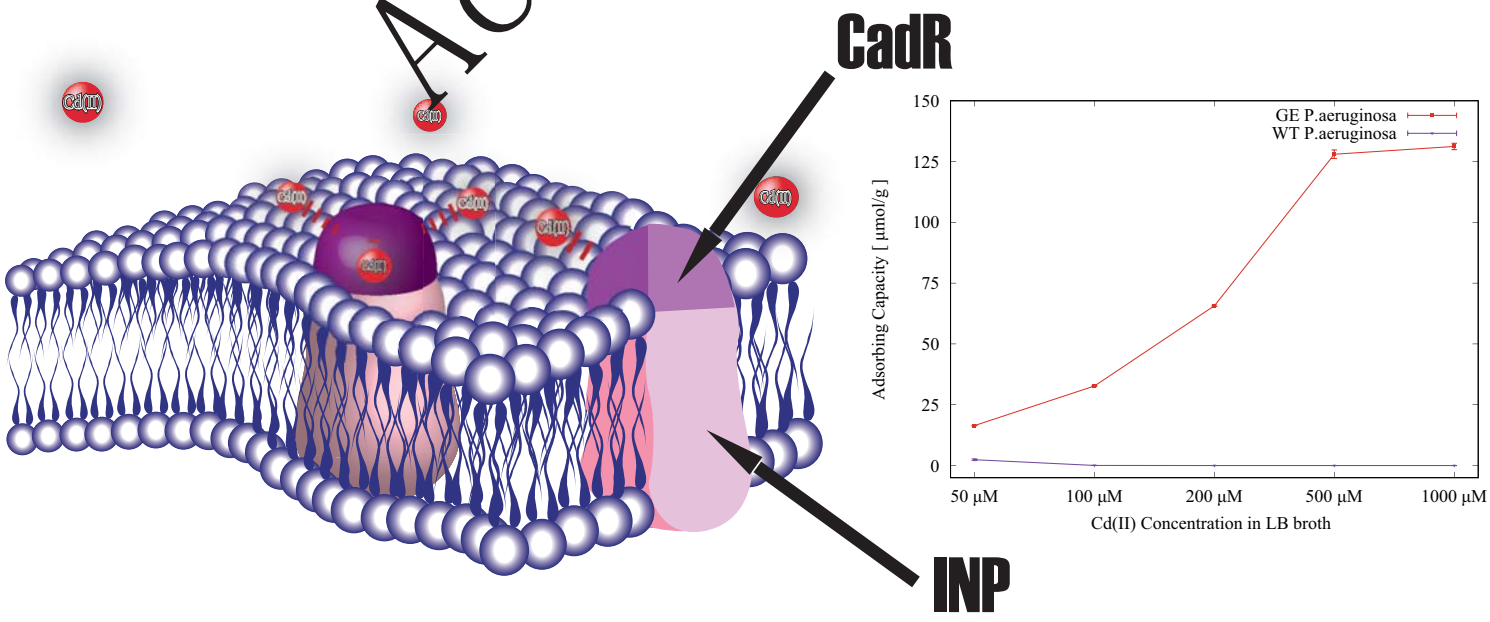
lized phanerochaete chrysosporium : Equilibrium, kinetic, thermodynamic and mechanisms analysis. Chemical Engineering Journal 203 (5), 423–431.

Yang, C., Chen, H., Zeng, G., Yu, G., Luo, S., 2010. Biomass accumulation and control strategies in gas biofiltration. Biotechnology Advances 28 (4), 531–540.

Yin, K., Lv, M., Wang, Q., Wu, Y., Liao, C., Zhang, W., Chen, L., 2016. Simultaneous bioremediation and biodetection of mercury ion through surface display of carboxylesterase e2 from pseudomonas aeruginosa pa1. Water Research 103, 383.

Zhang, Y., Zeng, G. M., Tang, L., Chen, J., Zhu, Y., He, X. X., He, Y., Chem, A., 2015. Electrochemical sensor based on electrodeposited graphene-au modified electrode and nanoau carrier amplified signal strategy for attomolar mercury detection. Analytical Chemistry 87 (2), 989.

*Graphical Abstract



***Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)**

- We first express CadR that 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.

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

Xiang Tang[a,b], Guangming Zeng[a,b,*], Changzheng Fan[a,b,**], Man Zhou[a,b], Lin Tang[a,b], Jingjing Zhu[a,b], Jia Wan[a,b], Danlian Huang[a,b], Ming Chen[a,b], Piao Xu[a,b], Chen Zhang[a,b], Weiping Xiong[a,b]

[a]*College of Environmental Science and Engineering, Hunan University, Changsha 410082, China*
[b]*Key Laboratory of Environmental Biology and Pollution Control (Hunan University), Ministry of Education, Changsha 410082, China*

Abstract

Genetically engineered bacteria for pollution control of heavy metal have been widely studied, however, using *Pseudomonas aeruginos* (*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* was still flourished in the 30th generation in the LB broth which contained 100 μM Cd(II), exhibiting a excellent genetic stability. Chromosomal expressed *P.aeruginosa* showed a adsorption capacity of

*corresponding author. phone: +86-731-8-882370, fax: +86-731-8-8823701.
**corresponding author. phone: +86-13787102427.
Email addresses: zgming@hnu.edu.cn (Guangming Zeng), fancz@hnu.edu.cn (Changzheng Fan)

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.
Keywords: CadR, Chromosomal surface display, *Pseudomonas aeruginosa*, Cadmium, Bioremediation

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 bring 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 humans

health. Therefore, it is an urgent demand to seek for an efficient and environmentally compatible method to remove or detoxify heavy metals(Xu et al., 2012b; Chen et al., 2016; Song et al., 2017). Currently, the pollution treatment technologies contains 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 provides 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 ad-

sorption), 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 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 have 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 include plasmid expression and chromosome expression, which 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 applica-

tions(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 are comprised of 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 (1200-1500aa) and neither the C-terminal domain nor the central repeating domain (CRD) of the INPs harbours signal peptide sequences(Li et al., 2012). Therefore, the INP-N (the N-terminal domain) or the INP-NC (containing only the N-terminaland 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 the chromosome of *P.aeruginosa* by tripartite mating. This is the first use of *P.eruginosa* 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 gene 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 was synthetized by GENEWIZ. The *INP* and *cadR* were conjugated through touchdown-PCR: at first, Primer 1 (5'-AAGCTCTAGAGCATGAA CCGCGAGAAGGTGCTGGCCCT-3' (XbaI site underlined)) and Primer 2 (5'-GCCAGCTCGCCGATCTTCATGAACTCGATCTCGCGGGTG-3') was employed to amplify the INP coding gene; Primer 3 (5'-CACCCGCGAGATCGAGTTCATGA AGATCGGCGAGCTGGC-3') and Primer 4 (5'-AATATCGGGATCCCGGTGGCC GTGGCTGCGG CCCACGT-3' (BamHI site underlined)) was employed to amplify the *cadR* coding gene. In overlap PCR process, Primer 1 and Primer 4 was utilized to amplify the fused INP and *cadR* gene. The overlap PCR profile was as follows: 95 °C for 5 minutes; 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 minutes. 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)) was 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 was 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.

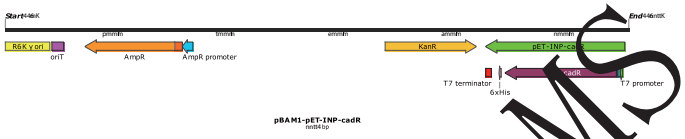


Figure 1: Schematic representation of the main features of the version of mini-Tn5 engineered in the pBAM1 backbone, containing the CadR coding gene fused with the INP encoding gene, and the *INP-cadR* insert to T7 promoter.

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 bacterias 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 hours 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 contains 100 μM cadmium and 50 μg /L kanamycin) in appropriate diluted concentration, then cultured in selective medium at 30 °C for 36 hours. 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 that containing 500 μM Cd(II) and 50 μg/mL of kanamycin, and the cultures was grown to an OD_{600} = 0.5-0.7 after 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 that containing 5% dry milk and allowed to incubated with anti-His Mouse monoclonal Antibody (CMCTAG) diluted in 1:2000 at room temperature for two hours. After washing three times with TBST (10 min for once), the blot was incubated with peroxidase-conjugated goat antimouse IgG (H+L)-HRP (CMCTAG) at 1:5000 dilution in blocking buffer for one hour. 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 hours by gentle shaking, and then, 10 L of the cultures was added to 1.0 mL fresh selective LB broth for 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 hours 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 five minutes at room temperature,and resuspended in 1 mL PBS buffer and 10 μL cultures before incubated to LB broth that 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 hours with 120 rpm shaking. After incubation, the cells were separated from the reaction solution by centrifugation at 5000 rpm for ten minutes 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 (AAS). 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 that 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 hours. 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 hours. 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 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 hours. 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 hours. Afterwards, monoclonal colony were inoculated to Cd(II)-contained LB broth and incubated at different temperature with for 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 spe-

cific to the absorption of heavy metals. When there are several kinds of metal ions in the environment of existence, competitive target of heavy metal ions of non target metal ions will be in metal binding sites, the target of heavy metal ions for binding site affinity than the target metal ions will occupy the preferred 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 presence of 981 bp fragment has initial verified that overlap PCR had undergone as expected (E-supplement). The pBAM1 have the mini-Tn5 transposon that can randomly inserted 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 initial verified by primer T7-F and primer T7-R, original T7 promoter gene was 380 bp, the recon-

structed 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 displaying 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) was close to their theoretical values.

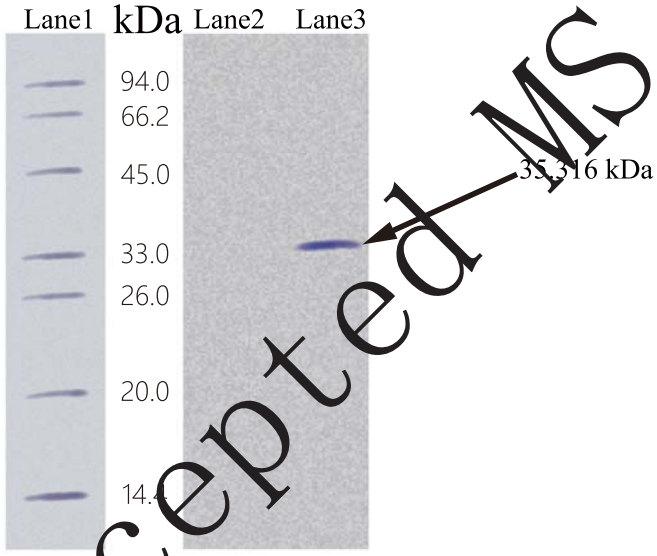


Figure 2: Western blot analysis of CadR. Lane 1, Protein standards. Lane 2, WT *P. aeruginosa*. Lane 3, GE *P. aeruginosa*.

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* show high tolerance to Cd(II) than *E.coli BL21(DE3)* that 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-replication in *E.coli BL21(DE3)* without the λpir protein(Martłnez-Garcła 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 were essential for their application in natural polluted environment. To examine whether chromosomal expression have 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 have shown the OD_{600} of selective LB broth after 12 hours gentle shaking at 30 °C in the Pass-generation assay. In *Con E.coli* group, the OD_{600} was consistently de-

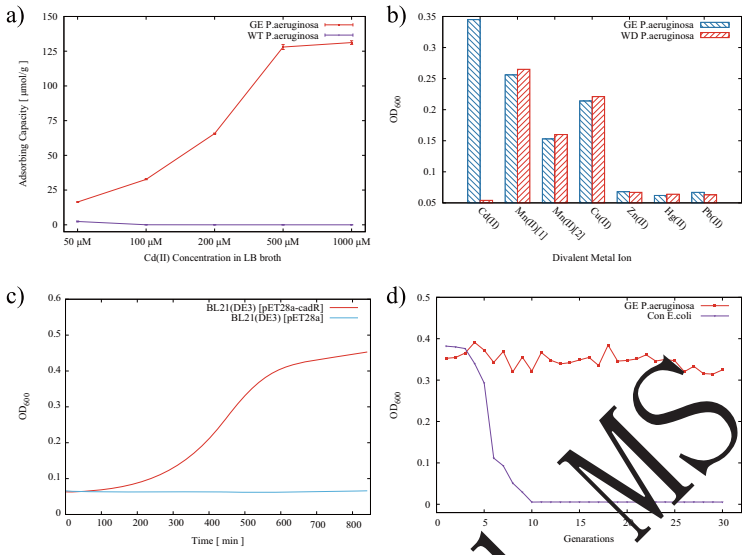


Figure 3: a) The plasmid pET-*cadR* and plasmid pET28a was transformed to *E.coli BL21(DE3)* compent cells, and its growth curve in LB broth (500 μM/L) was tested. b) The OD600 of GE *P. aeruginosa* when inoculated to LB broth under different divalent metal stress after 8 hours gentle shaking. c) Cd(II) 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 hours gentle shaking in 30°C in the Pass-generation assay.

creasing 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 *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 were 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 were 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 were 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 were 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 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 was 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 that gathering on the surface of WT *P.aeruginosa* that low than 50

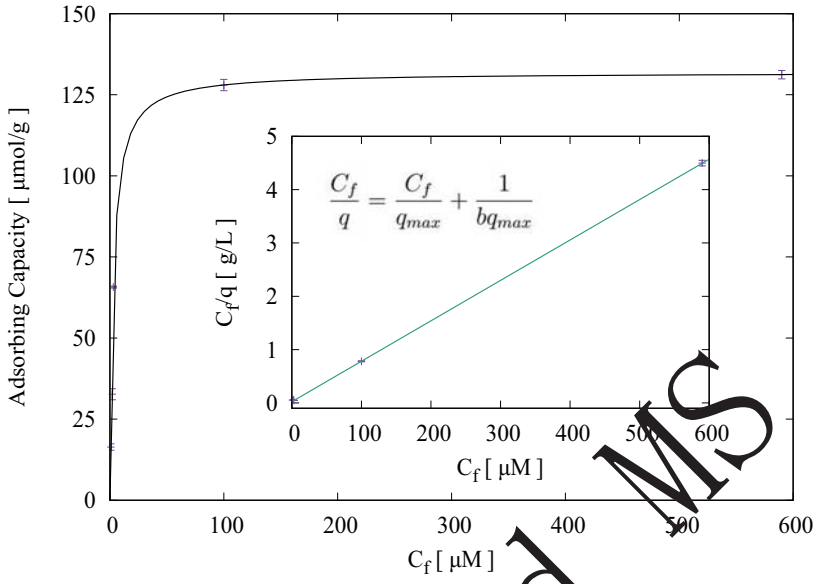


Figure 4: Sorption isotherm of Cd(II) by GE *P.aeruginosa* bacteria. Insert: Langmuir transformation for the sorption isotherm of Cd(II).

μM and cannot grown 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, non-specific 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 high selectivity towards Cd(II) over Mn(II), Cu(II), Zn(II), Hg(II) and Pb(II), and the WT *P.aeruginosa* have 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 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, acid environment was inhibit the cell activity.

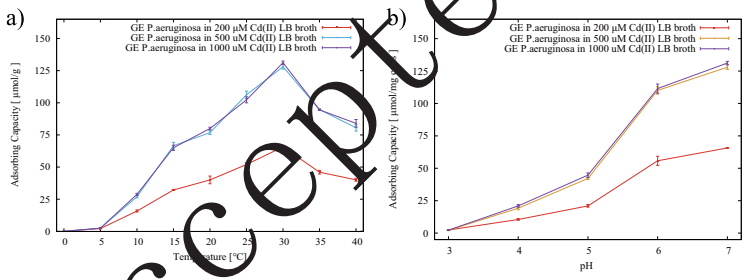


Figure 5: a) Cd(II) adsorption capacity of GE *P. aeruginosa* in different pH. b) Cd(II) adsorption capacity of GE *P. aeruginosa* in different temperature.

When GE *P.aeruginosa* cultivated in gradient temperature, GE *P.aeruginosa* have 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* have 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).

acknowledgement

The study was fnancially supported by the National Natural Science Foundation of China (51521006, 51679084, 51378190, 51579096, 51508177, 51709101)

385 and the Program for Changjiang Scholars and Innovative Research Team in Uni-
386 versity (IRT-13R17).

387 Baldwin, D. R., Marshall, W. J., 1999. Heavy metal poisoning and its laboratory
388 investigation. Annals of Clinical Biochemistry 36 (3), 267–300.

389 Brocklehurst, K., Megit, S., Morby, A., 2003. Characterisation of cadr from pseu-
390 domonas aeruginosa: a cd (ii)-responsive merr homologue. Biochemical and
391 biophysical research communications 308 (2), 234–239.

392 Brown, N. L., Stoyanov, J. V., Kidd, S. P., Hobman, J. L., 2003. The merr family
393 of transcriptional regulators. FEMS microbiology reviews 27 (2-3), 145–163.

394 Chen, J., Sun, G.-X., Wang, X.-Y., Lorenzo, V. d., Rosen, B. P., Zhu, Y.-G., 2014.
395 Volatilization of arsenic from polluted soil by pseudomonas putida engineered
396 for expression of the arsm arsenic (iii) s-adenosine methyltransferase gene. En-
397 vironmental science & technology 48 (17), 10337–10344.

398 Chen, M., Xu, P., Zeng, G., Yang, C., Huang, D., Zhang, J., 2015. Bioremediation
399 of soils contaminated with polycyclic aromatic hydrocarbons, petroleum,
400 pesticides, chlorophenols and heavy metals by composting: Applications,
401 microbes and future research needs. Biotechnology Advances 33 (6, Part 1),

745 – 755.

URL http://www.sciencedirect.com/science/article/pii/S0734975015300021

Chen, Z., Cui, L., Zeng, G., Huang, D., Lin, T., Yang, C., Zhou, Y., Lei, Q., Min, C., 2016. Nanoporous au-based chronocoulometric aptasensor for amplified detection of pb 2+ using dnazyme modified with au nanoparticles. Biosensors & Bioelectronics 81, 61.

Cheng, Y., He, H., Yang, C., Zeng, G., Li, X., Chen, H., Yu, G., 2016. Challenges and solutions for biofiltration of hydrophobic volatile organic compounds. Biotechnology Advances 34 (6), 1091.

Chiu, T.-Y., Chen, P.-H., Chang, C.-L., Yang, D.-M., 2013. Live-cell dynamic sensing of cd2+ with a fret-based indicator. PloS one 8 (6), e65853.

Chungjatupornchai, W., Fa-aroonsawat, S., 2009. Translocation of green fluorescent protein to cyanobacterial periplasm using ice nucleation protein. The Journal of Microbiology 47 (2), 187–192.

Cui, J., Liu, T., Li, F., Yi, J., Liu, C., Yu, H., 2017. Silica nanoparticles alleviate cadmium toxicity in rice cells: Mechanisms and size effects. Environmental Pollution 228 (Supplement C), 363 – 369.

URL http://www.sciencedirect.com/science/article/pii/S0269749116318486

420 Fan, L.-H., Liu, N., Yu, M.-R., Yang, S.-T., Chen, H.-L., 2011. Cell surface dis-
421 play of carbonic anhydrase on escherichia coli using ice nucleation protein for
422 co2 sequestration. Biotechnology and bioengineering 108 (12), 2853–2864.

423 Gong, J. L., Wang, B., Zeng, G. M., Yang, C. P., Niu, C. G., Niu, Q. Y., Zhou,
424 W. J., Yi, L., 2009. Removal of cationic dyes from aqueous solution using mag-
425 netic multi-wall carbon nanotube nanocomposite as adsorbent. Journal of Haz-
426 ardous Materials 164 (2C3), 1517–1522.

427 Huang, C. C., Hsieh, J. L., Su, C. C., Tseng, C. F., Lin, P. J., Chang, J. S., 2003.
428 Polypeptides for heavy-metal biosorption:capacity and specificity of two het-
429 erogeneous merp proteins. Enzyme & Microbial Technology 33 (4), 379–385.

430 Khodi, S., Latifi, A. M., Saadati, M., Mirzaei, M., Aghamollaei, H., 2012. Surface
431 display of organophosphorus hydrolase on e. coli using n-terminal domain of
432 ice nucleation protein inav. Journal of Microbiology & Biotechnology 22 (2),
433 234.

434 Kozloff, L., Turner, M., Arellano, F., 1991. Formation of bacterial membrane ice-
435 nucleating lipoglycoprotein complexes. Journal of bacteriology 173 (20), 6528–
436 6536.

437 Lee, S.-W., Glickmann, E., Cooksey, D. A., 2001. Chromosomal locus for cad-

mium resistance in pseudomonas putida consisting of a cadmium-transporting atpase and a merr family response regulator. Applied and environmental microbiology 67 (4), 1437–1444.

Li, Q., Yan, Q., Chen, J., He, Y., Wang, J., Zhang, H., Yu, Z., Li, L., 2012. Molecular characterization of an ice nucleation protein variant (inaq) from pseudomonas syringae and the analysis of its transmembrane transport activity in escherichia coli. International journal of biological sciences 8 (8), 1097.

Li, Q., Yu, Z., Shao, X., He, J., Li, L., 2009. Improved phosphate biosorption by bacterial surface display of phosphate-binding protein utilizing ice nucleation protein. FEMS microbiology letters 299 (1), 44–52.

Liu, Q., Yuan, F., Liang, Y., Li, Z., 2015. Cadmium adsorption by e. coli with surface displayed cadr. RSC Advances 5 (21), 16089–16092.

Long, F., Gong, J. L., Zeng, G. M., Chen, L., Wang, X. Y., Deng, J. H., Niu, Q. Y., Zhang, H. Y., Zhang, X. R., 2011. Removal of phosphate from aqueous solution by magnetic feczr binary oxide. Chemical Engineering Journal 171 (2), 448–455.

Malik, A., 2004. Metal bioremediation through growing cells. Environment International 30 (2), 261–78.

Martłnez-Garcła, E., Calles, B., Arvalo-Rodrłguez, M., de Lorenzo, V., 2011. pbam1: an all-synthetic genetic tool for analysis and construction of complex bacterial phenotypes. BMC Microbiology 11 (1), 38.

Ming, C., Qin, X., Zeng, G., Jian, L., 2016. Impacts of human activity modes and climate on heavy metal "spread" in groundwater are biased. Chemosphere 152, 439–445.

Ren, X., Zeng, G., Tang, L., Wang, J., Wan, J., Liu, Y., Yu, J., Yi, H., Ye, S., Deng, R., 2017. Sorption, transport and biodegradation - an insight into bioavailability of persistent organic pollutants in soil. Science of the Total Environment 610-611, 1154–1163.

Schmid, D., Pridmore, D., Capitani, G., Battistutta, R., Neeser, J.-R., Jann, A., 1997. Molecular organization of the ice nucleation protein inav from pseudomonas syringae. FEBS letters 414 (3), 590–594.

Smith, G. P., 1985. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228, 1315–1318.

Song, B., Zeng, G., Gong, J., Jie, L., Xu, P., Liu, Z., Yi, Z., Chen, Z., Min, C., Yang, L., 2017. Evaluation methods for assessing effectiveness of in situ reme-

diation of soil and sediment contaminated with organic pollutants and heavy metals. Environment International 105, 43.

Tan, X., Liu, Y., Zeng, G., Wang, X., Hu, X., Gu, Y., Yang, Z., 2015. Application of biochar for the removal of pollutants from aqueous solutions. Chemosphere 125 (Supplement C), 70 – 85.
URL http://www.sciencedirect.com/science/article/pii/S0045653514015008

Tang, W. W., Zeng, G. M., Gong, J. L., Liang, J., Xu, P., Zhang, C., Huang, B. B., 2014. Impact of humic/fulvic acid on the removal of heavy metals from aqueous solutions using nanomaterials: a review. Science of the Total Environment 468-469, 1014.

Tao, H.-C., Peng, Z.-W., Li, P.-S., Yu, T.-A., Su, J., 2013. Optimizing cadmium and mercury specificity of cadr-based e. coli biosensors by redesign of cadr. Biotechnology letters 35 (8), 1253–1258.

Wei, W., Liu, X., Sun, P., Wang, X., Zhu, H., Hong, M., Mao, Z. W., Zhao, J., 2014. Simple whole-cell biodetection and bioremediation of heavy metals based on an engineered lead-specific operon. Environmental Science & Technology 48 (6), 3363–71.

Wolber, P. K., Deininger, C. A., Southworth, M. W., Vandekerckhove, J.,

Van Montagu, M., Warren, G. J., 1986. Identification and purification of a bacterial ice-nucleation protein. Proceedings of the National Academy of Sciences 83 (19), 7256–7260.

Wu, C. H., Le, D., Mulchandani, A., Chen, W., 2009. Optimization of a whole-cell cadmium sensor with a toggle gene circuit. Biotechnology progress 25 (3), 898–903.

Wu, H., Lai, C., Zeng, G., Liang, J., Chen, J., Xu, J., Dai, J., Li, X., Liu, J., Chen, M., 2017. The interactions of composting and biochar and their implications for soil amendment and pollution remediation: a review. Critical Reviews in Biotechnology 37 (6), 754.

Xu, P., Zeng, G. M., Huang, D. L., Feng, C. L., Hu, S., Zhao, M. H., Lai, C., Wei, Z., Huang, C., Xie, G. X., Liu, Z. F., 2012a. Use of iron oxide nanomaterials in wastewater treatment: A review. Science of The Total Environment 424 (Supplement C), 1 – 10.
URL http://www.sciencedirect.com/science/article/pii/S0048969712001982

Xu, P., Zeng, G. M., Huang, D. L., Lai, C., Zhao, M. H., Wei, Z., Li, N. J., Huang, C., Xie, G. X., 2012b. Adsorption of pb(ii) by iron oxide nanoparticles immobi-

lized phanerochaete chrysosporium : Equilibrium, kinetic, thermodynamic and mechanisms analysis. Chemical Engineering Journal 203 (5), 423–431.

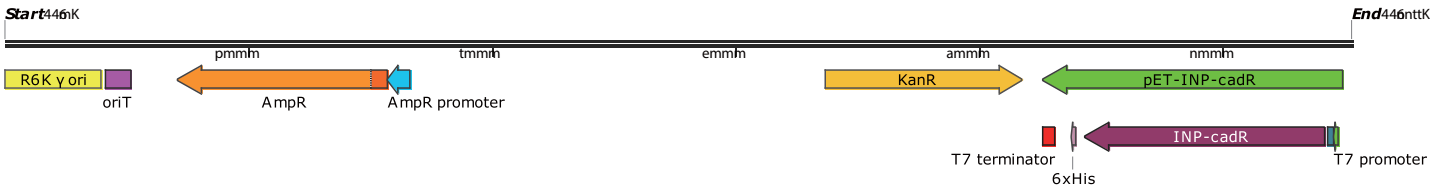
Yang, C., Chen, H., Zeng, G., Yu, G., Luo, S., 2010. Biomass accumulation and control strategies in gas biofiltration. Biotechnology Advances 28 (4), 531–540.

Yin, K., Lv, M., Wang, Q., Wu, Y., Liao, C., Zhang, W., Chen, L., 2016. Simultaneous bioremediation and biodetection of mercury ion through surface display of carboxylesterase e2 from pseudomonas aeruginosa pa1. Water Research 103, 383.

Zhang, Y., Zeng, G. M., Tang, L., Chen, J., Zhu, Y., He, X. X., He, Y., Chem, A., 2015. Electrochemical sensor based on electrodeposited graphene-au modified electrode and nanoau carrier amplified signal strategy for attomolar mercury detection. Analytical Chemistry 87 (2), 989.

Figure

Click here to download Figure: 1.eps



pBAM1-pET-INP-cadR
nntt4 bp

Figure
Click here to download Figure: 2.eps

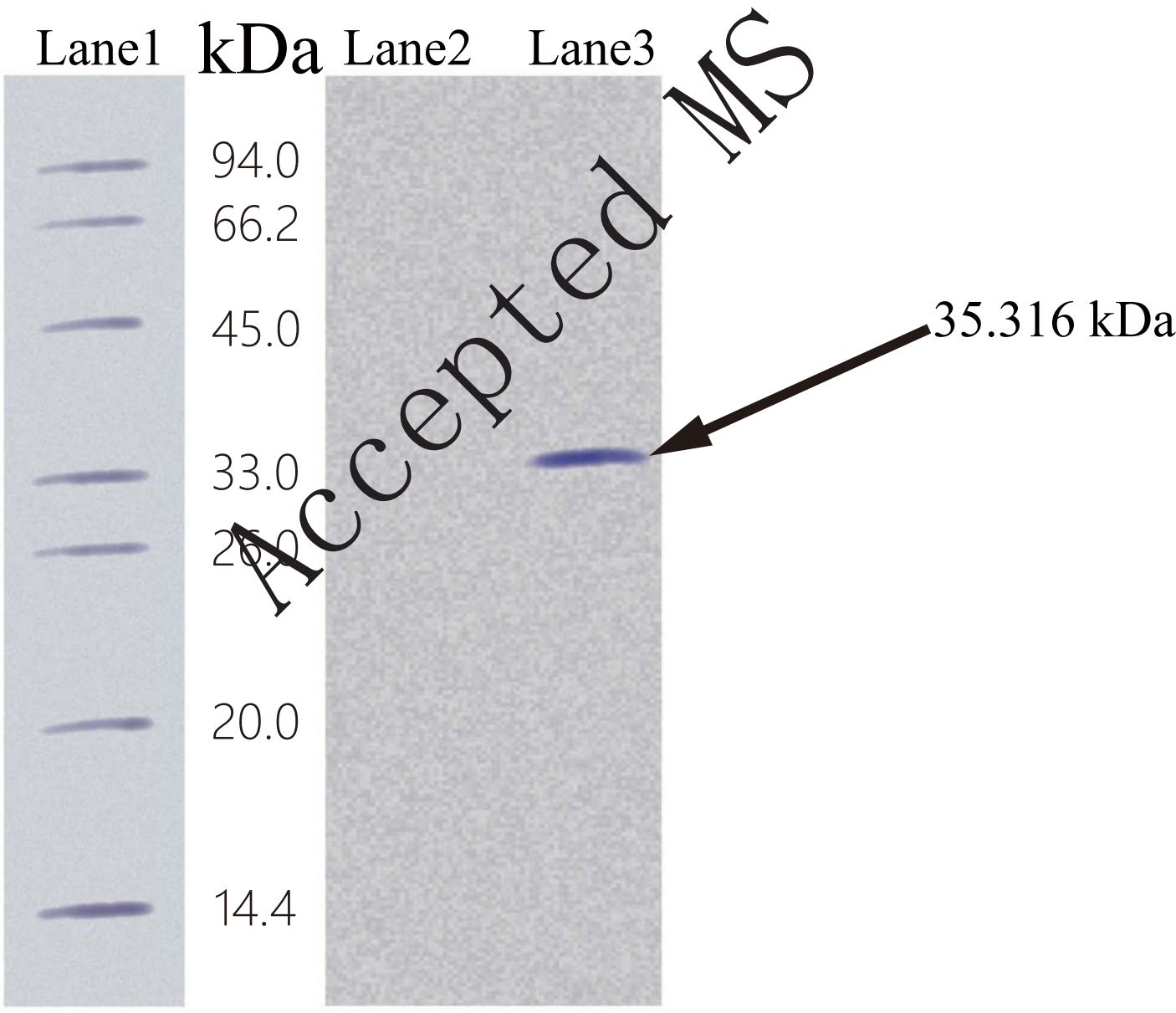
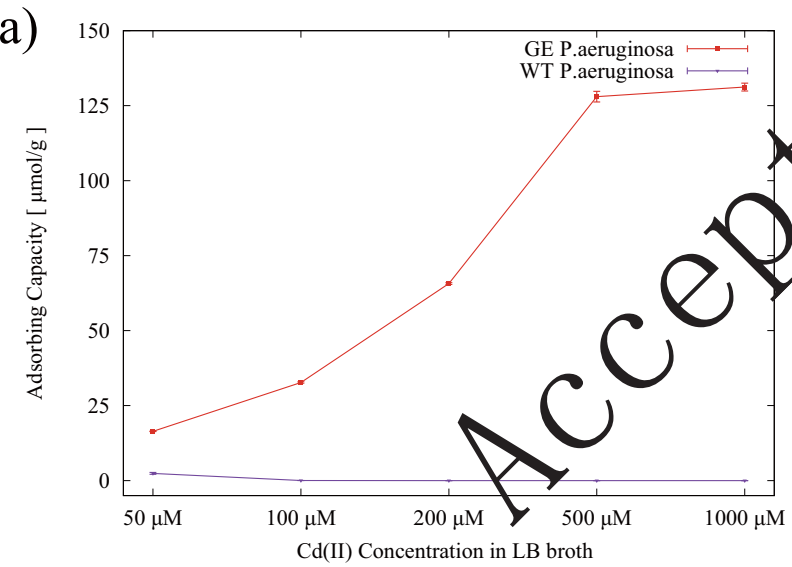
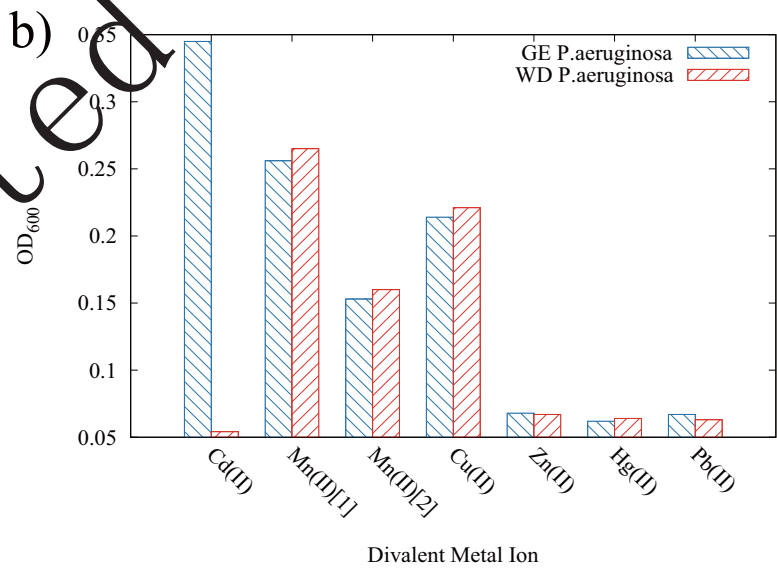
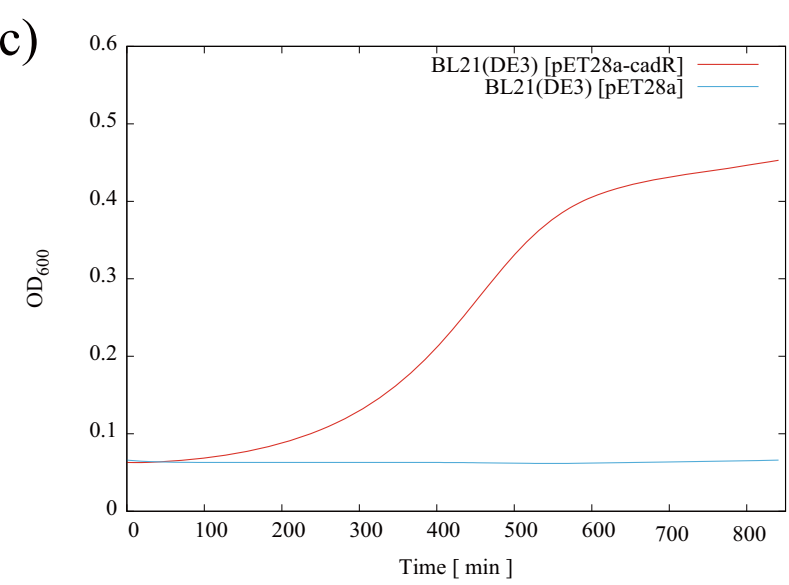


Figure
Click here to download Figure: 3.eps







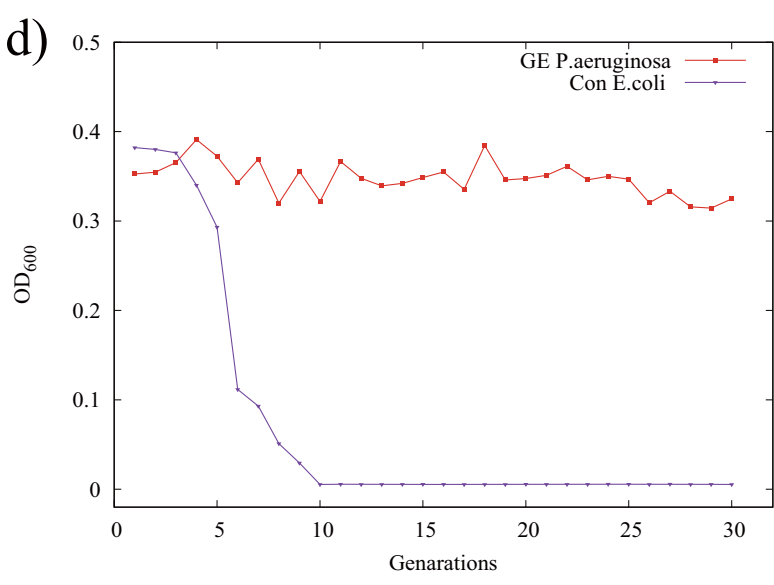


Figure
Click here to download Figure: 4.eps

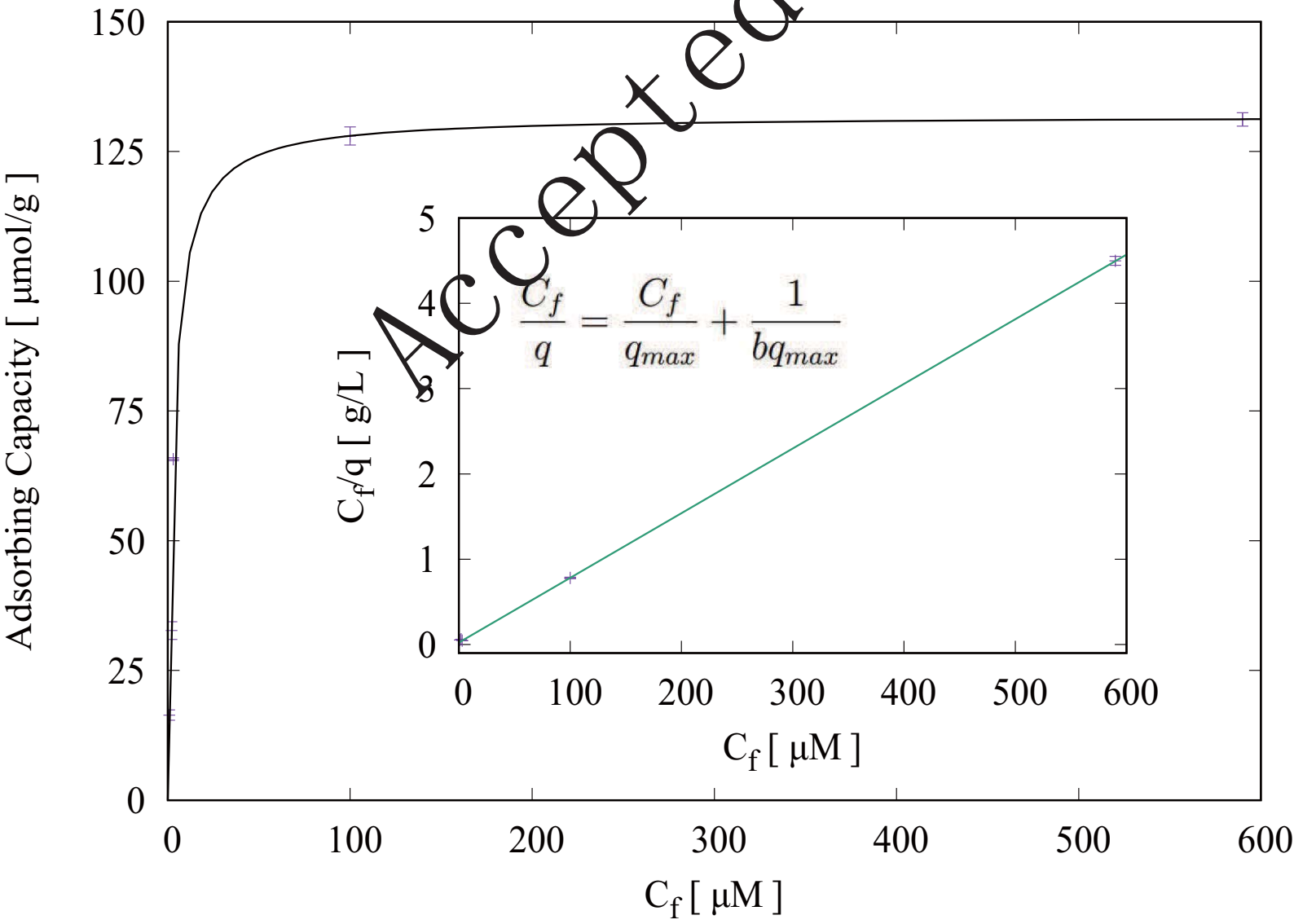


Figure
Click here to download Figure: 5.eps