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Identification of cadmium resistance and adsorption gene from *Escherichia coli* BL21 (DE3)†

Weitong Qin,‡ Xiaoqing Liu,‡ Xiaoxia Yu, Xiaoyu Chu, Jian Tian [ID] * and Ningfeng Wu

Cadmium is recognized as one of the most toxic heavy metals, and chronic cadmium exposure threatens plant, animal and human health. Because certain bacteria can play an important role in remedying heavy metal pollution, it is essential to further our understanding and clone cadmium adsorption and resistance genes from microorganisms. In this study, the cadmium resistance of different *Escherichia coli* strains including BL21 (DE3), JM109, DH5α, Top10 and Mach was determined. Among those strains, the *E. coli* BL21 (DE3) exhibited the highest cadmium resistance. Sequence analyses of four cadmium resistant clones screened from the BL21 (DE3) fosmid library showed that the insertion of each fosmid plasmid contained a fragment located at the lambda phage DE3 region. Next, we overexpressed several genes of this fragment in BL21 (DE3) and identified that cadmium resistance of *E. coli* is acquired through the *capB* gene. Indeed, overexpression of the *capB* gene increased the adsorption rate of cadmium. Taken together, these results indicated that the *capB* gene confers *E. coli* BL21 (DE3) with increased cadmium resistance by adsorption of cadmium. This study provided new insight into the mechanism of microbial cadmium resistance and expanded our understanding of cadmium resistance, adsorption and bioremediation by microorganisms.

1. Introduction

Cadmium is a nonessential nutrient element for organisms and is regarded as one of most toxic heavy metals with an unknown biological function.[1,2] Further, as a persistent toxic substance, cadmium pollution is irreversible and especially soil pollution is of great concern in many parts of the world.[3–6] Chronic cadmium exposure can not only affect the growth, yield and quality of the crops,[7] but cadmium can also enter the body through the food chain and consequently endanger human health; Itai-Itai disease, respiratory and kidney disease.[8,9]

According to previous studies, bacteria evolved several cadmium resistance mechanisms:[1,2,10–12] metal exclusion by a cellular permeability barrier such as the cell membrane or envelope; intracellular and extracellular sequestration through exopolysaccharide coating;[1–3] reduced metal sensitivity of cellular targets and transport efflux pumps.[13] Particularly the intracellular and extracellular binding of metal is of interest to researchers in the field of bioremediation as this method could be used to immobilize and adsorb target metals.

Escherichia coli, a common industrial Gram-negative bacterium and a model organism, is often used in studies concerning

Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China. E-mail: tianjian@caas.cn; Tel: +86-10-82109864

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‡ These authors contributed equally to this work.

microbial remediation of cadmium pollution.[14–21] Currently, most studies focus on the overexpression of cadmium resistant genes from Gram-positive bacteria to reverse cadmium pollution or to isolate strains in heavy metal contaminated areas:[2] *cadA* from *S. aureus*,[22] *czc* system from *Alcaligenes eutrophus*,[23] and *MTS*[16,23,24] and *phsABC*[25] from *Salmonella typhimurium*. In fact, *E. coli* is intrinsically tolerant to high levels of cadmium up to 0.9–1.0 mM.[16,25,26] However the exact mechanism of cadmium resistance in *E. coli* is not fully understood. To date, we know that ZntA, a Pb(II)-, Zn(II)-, and Cd(II)-transporting ATPases confer cadmium tolerance in *E. coli*.[13,17–19] Interestingly, most cadmium resistance mechanisms currently known in microbiology are cadmium exclusion. Thus, the identification of cadmium resistant genes, especially cadmium adsorption genes, in *E. coli* would be of great importance for applications in remedying cadmium contamination.

In this study, we determined the cadmium resistance of different strains of *E. coli* including BL21 (DE3), JM109, DH5α, Top10, and Mach. We discovered that BL21 (DE3) had the highest cadmium resistance, up to 1.0 mM. Consequently, we constructed a BL21 (DE3) fosmid library to look for genes conferring the high resistance of *E. coli* to cadmium. Four clones with higher cadmium resistance than the wild type were identified, and the insertion fragments of the four strains were overlapped. Through overexpression of different genes in this section, we were eventually able to identify the gene related to cadmium resistance and absorption. Based on our knowledge, this is the first study to report the key gene related to cadmium

absorption of BL21 (DE3). This work has paved the way for bioremediation and increasing cadmium resistance.

2. Material and methods

2.1 Bacterial strains and growth conditions

The plasmids and bacterial strains used in this study are listed in Table 1; EPI300-T1R came with the Fosmid Library Construct Kit. All the strains were grown in Luria Bertani (LB) medium ($10 g L^{-1}$ trypoton, $5 g L^{-1}$ yeast extract, $10 g L^{-1}$ NaCl) overnight at 37 °C with shaking at 200 rpm. All reagents used in this study were purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2 Determination of minimum inhibitory concentration (MIC) of cadmium

The MIC values[27] were assessed by measuring the growth rate of different strains estimated from the optical density at 600 nm (OD_{600}). For the assay, the overnight culture of the test strains was transferred into fresh LB medium and incubated at 37 °C until an OD_{600} of 0.6–0.8 was reached. Next, the different strains (1%) were inoculated into 96-well plates containing different concentrations of $CdCl_2$ (0–1.4 mM) in triplicate. After incubation at 37 °C for 20–24 hours, the OD_{600} was measured with a fluorescence absorbance cuvette.

2.3 Construction and screening of BL21 (DE3) genomic fosmid library

The BL21 (DE3) total genomic DNA was extracted with the Bacterial Genome Rapid Extraction Kit (BioTeKe, China), according to the manufacturer's protocol. DNA concentration and quality were checked by absorbance cuvette ($A_{260}/A_{280} \sim 1.8$) and gel electrophoresis (1%). Approximately $0.5 \mu g \mu L^{-1}$ DNA extract was used to construct a fosmid library with the Copy-Control HTP Fosmid Library Production Kit and pCC1FOS Vector (Epicentre, Madison, WI, USA), according to the manufacturer's instructions. Twelve fosmid clones were randomly selected for evaluating the insert size by BamHI digestion and

agarose gel electrophoresis analysis (Fig. S1†). A total of 1443 fosmid clones (insert size range 25–40 kb) were obtained and stored at −70 °C in 40% glycerol.

To screen for clones with cadmium resistance, all fosmid clones were replicated in 96-well plates containing LB broth supplemented with chloramphenicol ($12.5 \mu g mL^{-1}$) and cultivated at 37 °C for 8–12 h. The cultures were then transferred onto solid LB medium supplemented with chloramphenicol ($12.5 \mu g mL^{-1}$) containing 1.2 mM $CdCl_2$ and incubated at 37 °C for 36 h.

Fosmid DNA was isolated from the positive fosmid clones with the High Purity Plasmid Extraction Kit (TIANGEN, China) and sequenced from both ends with an ABI 3730 DNA analyzer and the pCC1FOS vector universal primers fosmid-T7 and fosmid-RP (Table. S1†). The sequences were analyzed by BlastN (NCBI) to determine the location of the inserted fragments in the entire genome.

2.4 Gene overexpression in E. coli

Genes containing their promoters predicted using Web Promoter Scan Services (https://www-bimas.cit.nih.gov/molbio/proscan/) were amplified by PCR from the positive plasmids with the primers listed in Table S1,† and digested with KpnI and XbaI. These fragments were inserted into the KpnI/XbaI double digest of pUC19-GFP, containing a reporter green fluorescent protein (GFP) gene gfp, to create plasmids pUC19-capA-GFP, pUC19-capB-GFP, pUC19-capJ-GFP, pUC19-regI-GFP, pUC19-regII-GFP and pUC19-regIII-GFP (Table. S1†). Each plasmid was verified by DNA sequencing and transformed into E. coli BL21 (DE3) to express the GFP-tagged proteins CapA, CapB, CapJ, CapC, CapD, CapE, CapFi, CapFii, CapV, CapQ and CapT, respectively. All the host cells harboring the recombinant vectors were grown in LB at 37 °C for 20 h. To confirm gene expression, fluorescence of the cultures was measured with a fluorescence absorbance cuvette. Fluorescence in the wells was measured at an excitation/emission wavelength of 484/507 nm, using a microplate reader SpectraMax M2 (Molecular Devices, USA).

Table 1 The plasmids and bacterial strains used in this study

Strains and plasmids	Description	Resources
BL21 (DE3)	F-,ompT,hsdS(rBB-mB-),gal,dcm(DE3)	CWBIO
DH5α	F-,φ80dlacZΔM15,Δ(lacZYA-agF)U169,deoR,ecA1, endA1,hsdR17(k−,mk+),phoA,supE44,λ-,thi-1,gyA96,elA1	CWBIO
JM109	ecA1,endA1,gyA96,thi-1,hsdR17,supE44,elA1, Δ(lac-poAB)F'[taD36,poAB+,lacIq,lacZΔM15]	CWBIO
Top10	F-,mcaAΔ(m-hsdRMS-mcBC),φ80,lacZΔM15, Δlacx74,ecA1,aaΔ139Δ(aa-leu)7697,galU,galK,ps,(St)endA1,nupG	Our lab
Mach	recA,tonA,endA1,hsdR, lacZΔM15	ThermoFisher
BL21-pUC19-GFP	Control strain with empty pUC19-GFP plasmid	Our lab
BL21-pUC19-capA-GFP	capA gene was overexpressed	In this study
BL21-pUC19-capB-GFP	capB gene was overexpressed	In this study
BL21-pUC19-capJ-GFP	capJ gene was overexpressed	In this study
BL21-pUC19-regI-GFP	I region was overexpressed	In this study
BL21-pUC19-regII-GFP	II region was overexpressed	In this study
BL21-pUC19-regIII-GFP	III region was overexpressed	In this study
pUC19-GFP	Expression vector	Our lab

BL21 (DE3) cells harboring the empty pUC19-GFP expression vector were used as the negative controls.

2.5 Cd²⁺ removal by protein CapB

The *capB* overexpression strains and control strains (BL21 (DE3)) with empty vector pUC19-GFP were cultivated in LB broth supplemented with ampicillin (100 μg mL^{-1}) for 12 h in a shaking incubator (200 rpm), before transferring them into 3 mL LB supplemented with 0.1 mM Cd²⁺ or 0.05 mM Cd²⁺ for 24 h. Then the cultures were transferred into 1.5 mL tubes and centrifuged at 10 000g for 10 min. The supernatant was separated and diluted 100 folds using 0.6 M HCl and the Cd²⁺ concentration was determined with an atomic absorption spectrophotometer (AAS).[28,29] Based on the mean absorbance, concentration of Cd²⁺ was determined by comparing with mean absorbance of the standard Cd²⁺. The experiments were performed in triplicate.

3. Results and discussion

3.1 Resistance of *E. coli* stains to Cd²⁺

To determine the degree of Cd²⁺ resistance of *E. coli*, we examined the growth of different *E. coli* stains including BL21 (DE3), JM109, DH5α, Top10 and Mach in LB liquid medium with various Cd²⁺ concentrations. As depicted in Fig. 1a, the strain BL21 (DE3) resisted Cd²⁺ concentrations of up to 1.0 mM. Strain Top10 resisted up to 0.8 mM Cd²⁺ and Mach, JM109 and DH5α up to 0.6 mM Cd²⁺. Clearly, from all the *E. coli* stains used in this study, BL21 (DE3) had the highest cadmium resistance (1.0 mM ± 0.02). The evident difference between BL21 (DE3) and the other four genetically engineered *E. coli* stains is that BL21 (DE3) carries a lysogenic lambda phage DE3 that expresses T7 RNA polymerase under the control of a lacUV5 promoter.[30] So we speculated that this specific DE3 sequence, located at 748 396–791 320 in the BL21 (DE3) genome, might confer the higher resistance to cadmium.

3.2 Screening of the Cd²⁺ resistant DNA fragments

To figure out the gene(s) involved in the Cd²⁺ resistance of BL21 (DE3), a fosmid library from genomic DNA of BL21 (DE3) was constructed in the *E. coli* strain EPI300-T1R and screened for Cd²⁺ resistant clones. Out of the 1443 clones, four clones (1mMCd-1, 1mMCd-2, 1mMCd-3 and 1mMCd-4) exhibited higher Cd²⁺ resistance than the host strain EPI300-T1R (Fig. 1b). The fosmid DNA isolated from these four clones was sequenced and by aligning the sequences with the BL21 (DE3) genome (accession number: NC_012892), we found that the inserted fragment from the four plasmids all contained a DNA fragment located between 768 481 and 801 476 (Table 2). Indeed, this region overlapped with the aforementioned DE3 sequence. The result confirmed that the DE3 sequence is important for the cadmium resistance of BL21 (DE3).

3.3 Overexpression of protein CapB can improve the cadmium resistance of BL21 (DE3)

To identify the cadmium resistant gene(s) in the DE3 sequence, we further analyzed the genetic composition and gene function of the fragment locating between 768 481 and 791 320. It contained 24 genes coding for DNA packing proteins, capsid components and tail components (Fig. 2). In order to identify the target genes conferring the cadmium resistance, some of these 24 genes were overexpressed in BL21 (DE3) to see whether they could increase the cadmium resistance of BL21 (DE3). The selected genes were fusion expressed with GFP to conveniently detect the expression of target genes by measuring fluorescence. As shown in Fig. 3a, the genes *capA*, *capB* and *capJ* were successfully overexpressed. However, only overexpression of *capB* could increase the MIC of Cd²⁺ for BL21 (DE3) from 1.0 mM to 1.2 mM (Fig. 3b). In order to confirm that the CapB

Table 2 The location of the insertion of the different plasmids in the BL21(DE3) genome

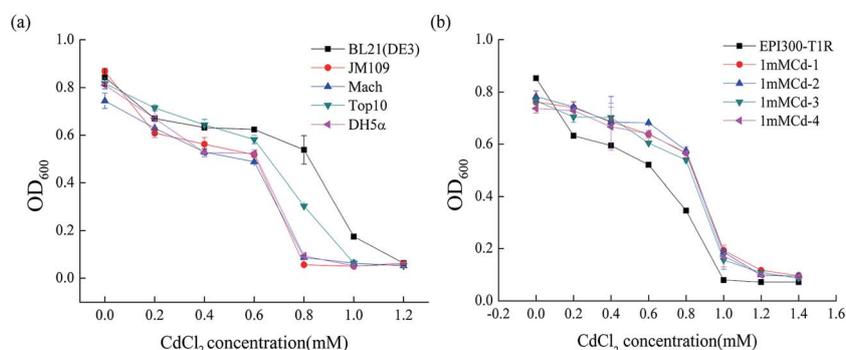| Plasmid number	The location of the insertion in BL21 (DE3) genome	Overlap section
1mMCd-1	768 481–825 247	768 481–801 476
1mMCd-2	768 435–801 476	
1mMCd-3	768 464–823 194	
1mMCd-4	768 403–824 390	

Fig. 1 (a) The minimum inhibitory concentration (MIC) of different strains of *E. coli* against cadmium. (b) The MIC of anti-cadmium strains screened in the fosmid library against cadmium. EPI300-T1R, the control strain from the Fosmid Library Production Kit; 1mMCd-1, 1mMCd-2, 1mMCd-3, 1mMCd-4 are strains screened in the fosmid library.
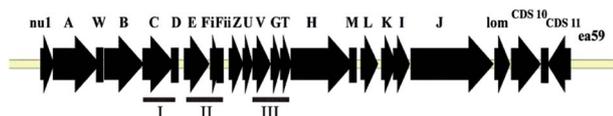
Fig. 2 The sketch map of the overlapping DE3 sequence located at 768 481–791 320. It contains 24 genes, capA is shown as A; I (regI), II (regII), III (regIII) indicate the three regions that we overexpressed.

protein is expressed under its own promoter, we also performed SDS-PAGE (Fig. S2†). In addition, overexpression of *capB* in DH5α was also shown to improve the Cd^{2+} resistance of DH5α (Fig. 3c).

CapB encodes a capsid component of the lambda phage DE3 and the isoelectric point (pI) of this protein was 5.31. In the neutral pH environment in the cell, CapB has a negative charge that could bind to the positive ion of cadmium. Similarly, also the pIs of the proteins CapC, CapD, CapE, CapFi, CapFii, CapV, CapG and CapT were low (Table S2†). We therefore also selected these to be overexpressed in BL21 (DE3) in the hope to identify more Cd^{2+} resistance related genes. Based on the promoter and position of these genes, we expressed three fragments separately: region I (containing genes *capC* and *capD*), region II (containing genes *capE*, *capFi* and *capFii*) and region III

(containing genes *capV*, *capG* and *capT*) (Fig. 2). Although the proteins were successfully expressed in BL21 (DE3) (Fig. 3a), none of the fragments changed its Cd^{2+} resistance (Fig. 3c). Taken together, the results suggested that the CapB protein encoded by *capB* contributes to the Cd^{2+} resistance of *E. coli*.

3.4 Absorption of cadmium ions by the protein CapB

As CapB is not a membrane protein, we speculated that it may be expressed in cells to increase Cd^{2+} resistance by absorbing Cd^{2+}. To verify this, the final concentration of Cd^{2+} was measured in culture media supernatant after cultivation of *capB* overexpressing strains. We found that the Cd^{2+} removal rate of *capB* overexpressing strains was significantly higher than the wild type BL21 (DE3) strain (Fig. 4). At 0.05 mM Cd^{2+}, the Cd^{2+} removal rate of *capB* overexpressing strains was about two-fold greater than that of the wild type BL21 (DE3). At 0.1 mM Cd^{2+}, the Cd^{2+} removal rate of *capB* overexpressing strains was also higher than that of the wild type (Fig. 4). It was speculated that such a high concentration of metal ions was toxic to cells and the gradual saturation of adsorption sites by cadmium ions would have limited the capacity of Cd^{2+} removal.[31] The above results suggested that the CapB protein indeed increased cadmium resistance by absorbing more Cd^{2+}. Unlike the earlier
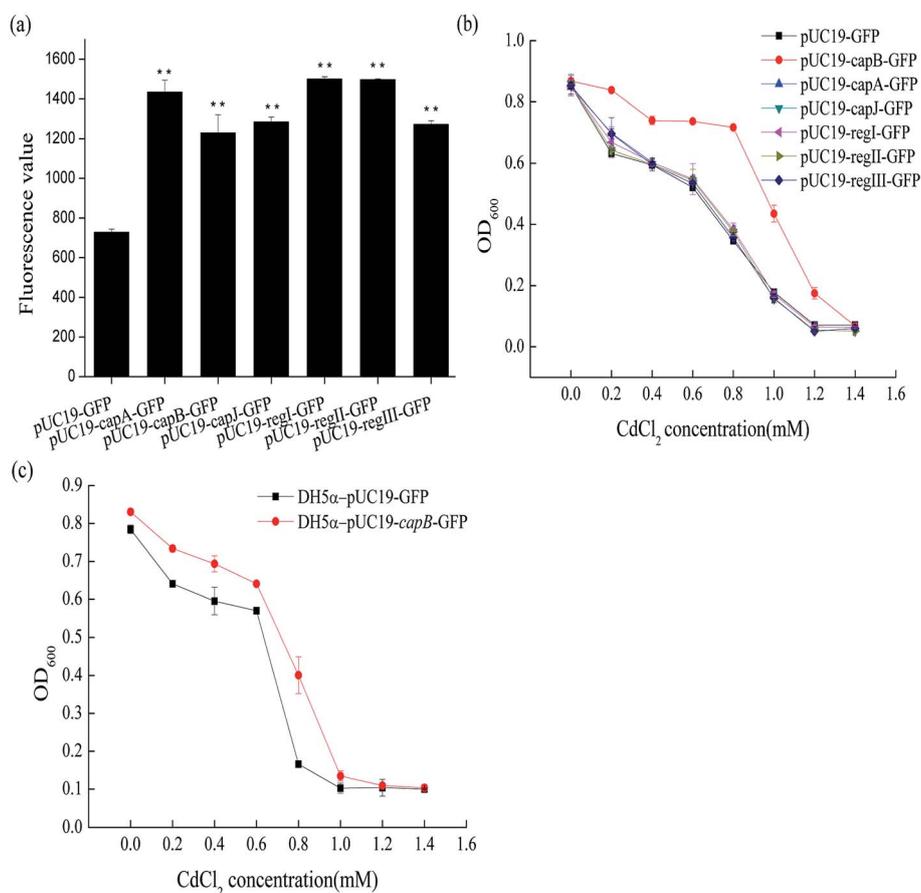

Fig. 3 (a) The fluorescence of different overexpressed genes in BL21 (DE3). pUC19-GFP is the negative control. **, $p < 0.01$. (b) The cadmium MIC value of *capA*, *capB*, *capJ*, regI, regII and regIII overexpressing recombinant strains (BL21 (DE3) as the host). BL21-pUC19 is the control strain. (c) The cadmium MIC value of the capB overexpressing recombinant strain (DH5α as the host). DH5α-pUC19 is the control strain.
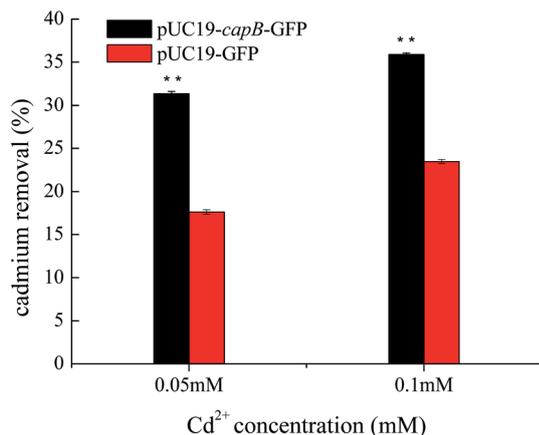
Fig. 4 Determination of cadmium adsorption capacity of recombinant strains with pUC19 as the expression vector. CapB overexpressing strains were cultivated at 0.05 mM and 0.1 mM Cd^{2+}, indicated as 0.05 mM and 0.1 mM respectively. pUC19-GFP was used as the control. **, $p < 0.01$. Ordinate represents the cadmium removal efficiency.

discovered metallothionein,[10,16,32,33] CapB does not contain a lot of cysteine short peptides. The pI of CapB (5.31) was lower than the neutral pH, implying that the protein surface will have many negative charges that can bind the positive cadmium ions in the cell. Perhaps it is just those functional negative groups that chelate with cadmium ions.[34] However, a low pI is just one of the characteristics of CapB since not all proteins with a low pI could adsorb cadmium. Therefore, in addition to the low pI, the special sequence or structure is also fundamental to conferring cadmium resistance. Our future studies will thus focus on the binding characteristics of the CapB protein.

Our results reiterated that bacterial proteins could improve heavy metal resistance or adsorption. With advances in bioinformatics and the newly acquired knowledge on CapB, we may be able to improve upon the design of such proteins to remedy heavy metal pollution in the future.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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