The construction of an engineered bacterium to remove cadmium from wastewater

S. Chang and H. Shu

ABSTRACT

The removal of cadmium (Cd) from wastewater before it is released from factories is important for protecting human health. Although some researchers have developed engineered bacteria, the resistance of these engineered bacteria to Cd have not been improved. In this study, two key genes involved in glutathione synthesis (gshA and gshB), a serine acetyltransferase gene (cysE), a *Thlaspi caerulescens* phytochelatin synthase gene (TcPCS1), and a heavy metal ATPase gene (TcHMA3) were transformed into *Escherichia coli* BL21. The resistance of the engineered bacterium to Cd was significantly greater than that of the initial bacterium and the Cd accumulation in the engineered bacterium was much higher than in the initial bacterium. In addition, the Cd resistance of the bacteria harboring gshB, gshA, cysE, and TcPCS1 was higher than that of the bacteria harboring gshA, cysE, and TcPCS1. This finding demonstrated that gshB played an important role in glutathione synthesis and that the reaction catalyzed by glutathione synthase was the limiting step for producing phytochelatins. Furthermore, TcPCS1 had a greater specificity and a higher capacity for removing Cd than SpPCS1, and TcHMA3 not only played a role in *T. caerulescens* but also functioned in *E. coli*.

Key words | cadmium, engineered bacterium, glutathione, phytochelatin

S. Chang
H. Shu (corresponding author)
Haikou Experimental Station,
Chinese Academy of Tropical Agricultural Sciences,
Haikou,
China
and
Key Laboratory of Hainan Banana Genetics and Breeding,
Haikou,
China
E-mail: *plantfood772@hotmail.com*

H. Shu
Department of Biology,
Zhengzhou University,
Zhengzhou,
China

INTRODUCTION

Cadmium (Cd) is a heavy metal that is highly toxic to humans. Specifically, Cd increases free radicals, promotes lipid peroxidation, and is carcinogenic (Chandra *et al.* 2001). Exposure to Cd is considered one of the main causes of prostate and lung cancer, kidney tubule damage, rhinitis, and bone fractures in humans (Bertin *et al.* 2006; Nawrot *et al.* 2006). The Itai Itai disease observed in Japan directly results from eating Cd-contaminated rice (Yamagata & Shigematsu 1970). With the development of industrial activities, a large amount of wastewater containing Cd has been released from factories (Jarup 2003). Most of this water flows into agricultural soils, resulting in pollution of the crops grown on the Cd-polluted soil (Wu *et al.* 2010). At least 13,330 ha of farmland have been contaminated by varying levels of Cd in China (Liu *et al.* 2009). This Cd enters the body when humans eat the crops that are grown in such soils (Bernard 2008). Thus, Cd poses a serious problem for safe food production and environmental conservation (Liu *et al.* 2009). Therefore, removing Cd from wastewater before it flows out of factories is important for protecting human health.

The most commonly used methods for removing Cd have historically included physical methods and chemical treatment (Sheng *et al.* 2004). Chemical treatments can be used to treat large quantities of water over a short period (Hashim *et al.* 2011). However, such methods are always expensive and can easily produce secondary pollution (Ahluwalia & Goyal 2007). For example, after lime is added into the wastewater, the extra lime in the water may be toxic for crops (Colella *et al.* 2012). Furthermore, chemical treatments are only used when Cd is present at high concentrations (Ahluwalia & Goyal 2007). These methods are often ineffective when Cd ions are present at micromolar concentrations (Ahluwalia *et al.* 2007). In contrast, biological adsorption using microorganisms is a much cheaper and greener method (Monachese *et al.* 2012). Some *Lactobacillus* and *Bifidobacterium* species can absorb Cd to a certain extent. However, the detailed mechanism of adsorption remains unclear (Halttunen *et al.* 2007). In plants and yeasts, Cd can be detoxified through sequestration by phytochelatins (Mendoza-Cozatl *et al.* 2011). After the *Arabidopsis thaliana* phytochelatin synthase gene (AtPCS) was expressed

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in *Escherichia coli*, the Cd content in the transformed bacteria increased nearly 20-fold (Sauge-Merle *et al*. 2003). In addition, bacteria over-expressing the yeast phytochelatin synthase gene (SpPCS), the *E. coli* serine acetyltransferase gene (cysE), and the glutamylcysteine synthase gene (gshA) were able to accumulate more Cd than the control (Wawrzynska *et al*. 2005). However, although these engineered bacteria have been developed, none of them have been applied to actual Cd removal from wastewater. The following could explain why these bacteria have not been used in practice: (1) although an engineered bacterial strain has been developed, a certain amount of time is required to construct a fermentation system; or (2) although these engineered bacteria can accumulate more Cd than the original strains, their tolerance to Cd has not been enhanced (Sauge-Merle *et al*. 2003; Wawrzynska *et al*. 2005). If the Cd tolerances of the engineered bacteria are sufficiently high, entrepreneurs and governments would likely utilize them for the removal of Cd from wastewater.

In this study, several engineered bacteria were developed. Among them, the Cd tolerance and accumulation of ABET3P were much higher than those of the original bacterium. This result occurred because the engineered ABET3P bacterium harbors gshA and gshB (two key genes involved in glutathione synthesis), a heavy metal ATPase gene (TcHMA3), a serine acetyltransferase gene (cysE), and a *Thlaspi caerulescens* phytochelatin synthase gene (TcPCS1). *T. caerulescens* is well known for its excellent Cd accumulation capacity (Milner & Kochian 2008). In addition, TcHMA3 is a determinant for the Cd-hyperaccumulating capacity of *T. caerulescens* (Ueno *et al*. 2011). The engineered bacterium strain ABET3P showed promising potential for removing Cd from real wastewater.

METHODS

Bacteria growth and culture conditions

Bacteria were grown in Luria-Bertani (LB) medium (Sambrook *et al*. 1989) containing kanamycin (50 μg/mL) or no kanamycin. The cultures were grown at 37 °C until they reached $OD_{600} = 1.8$. Then, 1 mL of these cultures were transferred into 100 mL of fresh LB medium and continually grown at 37 °C while shaking with a rotary shaker. β-d-1-thiogalactopyranoside (IPTG) was added into the cultures at a final concentration of 1 mmol/L when the OD_{600} was approximately 0.5. One hour later, $CdCl_2$ was added to the cultures at a final concentration of 0.5 mmol/L. The cadmium contents in the bacteria were measured after the bacteria were treated with Cd for 5 hours. For each trial, three replicates were carried out, and the averages were used. Chemical reagents were purchased from Sigma-Aldrich (Shanghai, China) unless otherwise specified. *E. coli* BL21 and MG1655 were purchased from Beinuo Biotech Company (Shanghai, China).

Plant growth and culture conditions

Seeds of *T. caerulescens* (Ganges ecotype) were donated by Professor Jiang (Liu *et al*. 2006). The seeds were germinated on moistened filter paper in a Petri dish and incubated at 22 °C in the dark for 1 week. Next, the seedlings were transferred to a 3.5-L plastic pot containing an aerated 1/5-strength Hoagland nutrient solution (Ueno *et al*. 2011). The plants were cultured in a temperature-controlled growth chamber at 22 °C (light intensity of 180 μmol/m^2/s, 14-hour day/10-hour night, and 60% relative humidity). The nutrient solution was renewed every 2 days during cultivation.

DNA cloning and plasmid construction

Standard molecular cloning techniques were performed as described by Sambrook *et al*. (1989). The total RNA from *T. caerulescens* was extracted using an RNeasy plant mini kit (Qiagen, Shanghai, China). The CDNA was prepared using a PrimeScript™ II 1st strand cDNA synthesis kit (Takara, Dalian, China). In addition, *E. coli* MG1655 genomic DNA was prepared using a quick bacteria genomic DNA extraction kit (Guangzhou Dongsheng Biotech Company, Guangzhou, China). Information regarding the primers used can be found in Table 1.

Table 1 | Primers used in this study (ribosome-binding sites are shown in italic letters. Restriction enzyme sites are underlined)

name	sequences
PTCPCS11	5′-CTC CGT CGA GGA TCC ATG GCT ATG GCG AGT TTG TAT C-3′
PTCPCS12	5′-TCG AGT GCG AAG CTT TCA AAA GGC AGG AGC AAC GAG A-3′
PTCPCS13	5′-TAA *AGG AGG* ACA GCT ATG GCT ATG GCG AGT TTG TAT C-3′
PTCPCS14	5′-AGT *AGG AGG* ACA GCT ATG GCT ATG GCG AGT TTG TAT C-3′
PGSHA1	5′-CTC CGT CGA GGA TCC ATG ATC CCG GAC GTA TCA CA-3′
PGSHA2	5′-ATC CCG *CCT CCT* TCA GGC GTG TTT TTC CAG CCA GC-3′
PGSHB1	5′-TGA AGG *AGG CGG* GAT ATG ATC AAG CTC GGC ATC GTG-3′
PGSHB2	5′-GTC AAA *CCT CCT* AGG TTA CTG CTG CTG TAA ACG TGC-3′
PCYSE1	5′-TGA AGG *AGG CGG* GAT ATG TCG TGT GAA GAA CTG GAA-3′
PCYSE2	5′-AGC TGT *CCT CCT* TTA GAT CCC ATC CCC ATA CTC AAA-3′
PCYSE3	5′-CCT *AGG AGG* TTT GAC ATG TCG TGT GAA GAA CTG GAA-3′
PTCHMA31	5′-TAA *AGG AGG* ACA GCT ATG GCG GAC GGT GAA GAG GCG-3′
PTCHMA32	5′-AGC TGT *CCT CCT* ACT TGT CTC CTC ACT CTT CTG CAG-3′

To construct the pTP plasmid, the PTCPCS11 and PTCPCS12 primers were used to amplify the TcPCS1 gene from *T. caerulescens* cDNA. The pET28a plasmid was digested with Bam HI and Hind III. The digested plasmid and the amplified TcPCS1 gene were mixed as a template. In-fusion cloning reactions were performed according to the In-Fusion HD Cloning Kit User Manual (Takara, Dalian, China). The competent *E. coli* BL21 cells were transformed with the reaction mixture, and the transformed bacteria were spread on LB plates containing 50 μg/mL kanamycin. Four clones were picked from the LB plate containing 50 μg/mL kanamycin and were cultured. The plasmids were extracted and sequenced. Next, the plasmid containing the identified sequence (GenBank accession number: AY540104) was named pTP, and the bacterial strain containing the pTP plasmid was named TP. The construction of the bacterial strains AEP, ABEP, and ABET3P were similar to that of TP. Detailed procedures can be found in the supplementary file (available online at http://www.iwaponline.com/wst/070/448.pdf).

Determination of cadmium in bacterial cells

Cellular cadmium was mainly measured by using a previously described method (Wawrzynska *et al*. 2005). The cells were collected by centrifugation (4000 rpm, 10 minutes), rinsed three times using LB medium without Cd, and dried for 24 hours (55 °C) in a laboratory oven (Wujiang Hengxin dryer company, Wujiang, China). The dried samples were suspended in 9 mL of 65% HNO_3 that was supplemented with 0.9 mL of 35% H_2O_2 and mineralized for 25 minutes (180 °C) in a laboratory microwave oven (ETHOSPLUS, Milestone, Italy). Then, the samples were cooled and 0.1 mL of 35% H_2O_2 was added. The Cd contents of the bacterial cells were measured using flame atomic absorption spectrophotometry (AA-6501, SHIMADZU, Tokyo, Japan) at 228.8 nm with a mixture of air and acetylene. All of the samples were measured in triplicate.

RESULTS AND DISCUSSION

Construction of engineered bacteria

Phytochelatins can chelate Cd ions (Hirata *et al*. 2005). The toxicity of phytochelatin-Cd was lower than that of the Cd ions. The synthesis of phytochelatins consists of two distinct steps: in step 1, the formation of r-Glu-Cys occurs simultaneously with the cleavage of glycine from glutathione and is catalyzed by r-glutamylcysteine synthase, which is encoded by gene gshA. In step 2, the r-Glu-Cys unit is transferred to glutathione or an oligomeric phytochelatin peptide. Step 2 is catalyzed by glutathione synthetase, which is encoded by the gshB gene (Hirata *et al*. 2005; Musgrave *et al*. 2013). Theoretically, if enough phytochelatins are present in the cell, the bacterium could accumulate more Cd and develop a higher resistance to Cd (Hirata *et al*. 2005). However, although the previously developed bacteria had high phytochelatin contents, their Cd resistances did not improve (Sauge-Merle *et al*. 2003). Furthermore, the resistances of the engineered bacterium to Cd were lower than those of the initial strain in some cases (Wawrzynska *et al*. 2005). This result potentially occurred because the concentration of glutathione (the material used for synthesizing phytochelatins) fell short of the cellular demand after the phytochelatins were over-produced. The metabolic pathways related to glutathione were affected, and the bacterial growth was inhibited. Therefore, the successful engineered bacterium should produce enough phytochelatin and glutathione.

According to the sequences of *E. coli* cysE (GenBank accession number EG10187), *E. coli* gshA (EG10418), *E. coli* gshB (EG10419), TcPCS1 (AY540104), and TcHMA3 polymerase chain reactions (Ueno *et al*. 2011), primers were designed and (PCRs) were performed. The amplified fragments were cloned into the pET28a vector, and the final plasmid was named pABET3P. To identify whether glutamine, the material for synthesizing phytochelatins, played an important role in the bacterial Cd tolerance, the plasmids pTP (i.e., pET28a harboring gene TcPCS1) and pAEP (i.e., pET28a harboring genes gshA, cysE, and TcPCS1) were developed. In addition, to verify that gshB plays a key role during the process of glutamine synthesis, the plasmid pABEP (i.e., the vector pET28a harboring genes gshA, gshB, cysE, and TcPCS1) was developed. The schematic maps of the plasmids are shown in Figure 1. A detailed procedure can be found in the materials and methods sections and in the supplementary file (available online at http://www.iwaponline.com/wst/070/448.pdf). The plasmids were identified by sequencing and the results confirmed that the plasmids were developed correctly with no error in the open-reading frames.

All of the genes described above were transcribed from a T7 polymerase-dependent promoter (Figure 1). Ribosome-binding sites were introduced upstream of the coding regions to enhance their translation efficiency (Table 1). In each plasmid, the ribosome-binding site that the first gene used was present in pET28a. To perform the in-fusion cloning reactions, the reverse primer of each target gene had an approximately 15-bp sequence that overlapped with the

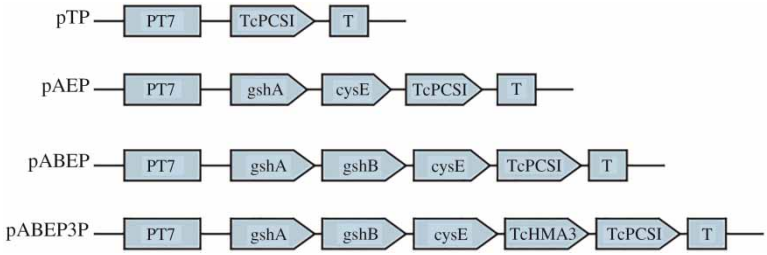

Figure 1 | Schematic map of the inserts present in the plasmids developed in this study. The basic plasmid vector was pET28a, and the PT7 had a T7 polymerase-dependent promoter. In addition, T represents the terminator.

forward primer of the adjacent gene (Table 1). Both the forward primer of the first gene and the reverse primer of the last gene in each plasmid had sequences of approximately 15-bp that overlapped with the pET28a vector (Table 1).

Growth rates of the *E. coli* strains cultured in medium without Cd

Generally, the growth curves of all strains showed an 'S' type. As shown in Figure 2, when the bacterial cells were cultivated for 8 hours, they entered the logistic phase. After being cultured for 11 hours, the OD_{600} of the strains was approximately 0.5. All growth curves showed similar trends until culturing for 11 hours, when the IPTG was loaded into the medium to induce the expression of the foreign genes. The OD_{600} values of ET28a and ABET3P were much higher than those of the other strains at each site after IPTG was loaded. For example, when they were cultivated for 17 hours, the OD_{600} values of ET28a and ABET3P were 2 and 1.9, respectively, whereas the OD_{600} values of TP, AEP, and ABEP were only 1.1, 1.2, and 1.4, respectively (Figure 2). No significant differences were observed between ET28a and ABET3P throughout the culturing process (Figure 2).

ABET3P had higher resistance to Cd than other strains

After the bacterial strains were cultured for 11 hours, 1 mmol/L IPTG was added into the LB liquid medium. After 1 hour, 0.5 mmol/L $CdCl_2$ was loaded into the cultures. Overall, the TP strain grew the slowest in the LB containing Cd, followed by the AEP strain. Although there was some evidence to suggest that these strains continued to grow after Cd was added the growth was very small. In contrast, the ABEP and ABET3P strains grew much better, and the ABET3P strain had the strongest resistance to Cd among the five strains (Figure 3). For example, after the bacterial cells were cultured for 17 hours, the OD_{600} of the ABET3P strain was 1.82, while the corresponding OD_{600} values of TP, AEP, ABEP, and ET28a were 0.73, 0.8, 1.2, and 1.68, respectively (Figure 3).

Cd accumulation in different bacteria strains

After culturing for 16 hours, the cells were collected, and the Cd content was measured. The quantity of Cd accumulated in the cells was the lowest in the ET28a strain, and the Cd content in the ABET3P cells was the highest (Figure 4). The cellular Cd content in the different strains can be

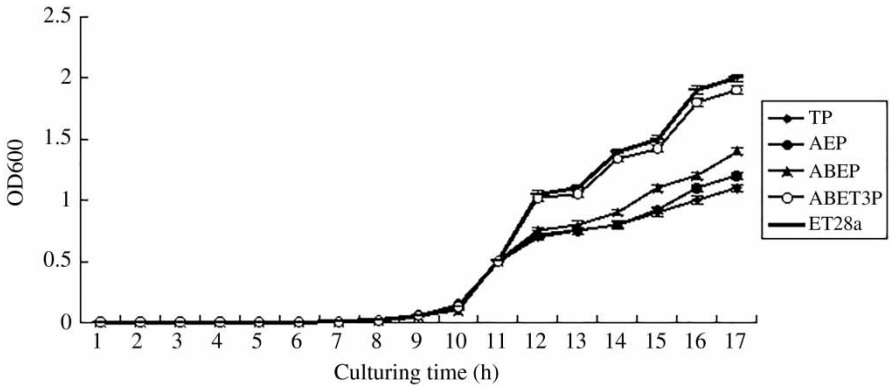

Figure 2 | Growth curves of *E. coli* BL21 strains harboring different plasmids in LB liquid medium without Cd.

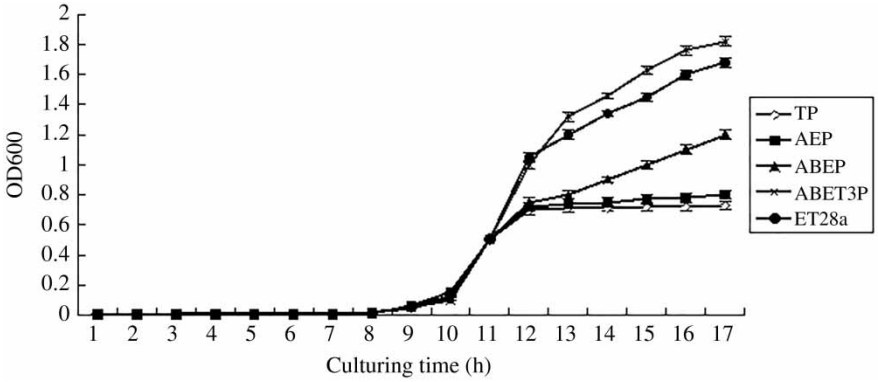

Figure 3 | Growth curves of different strains cultivated in LB supplied with 0.5 mmol/L $CdCl_2$.

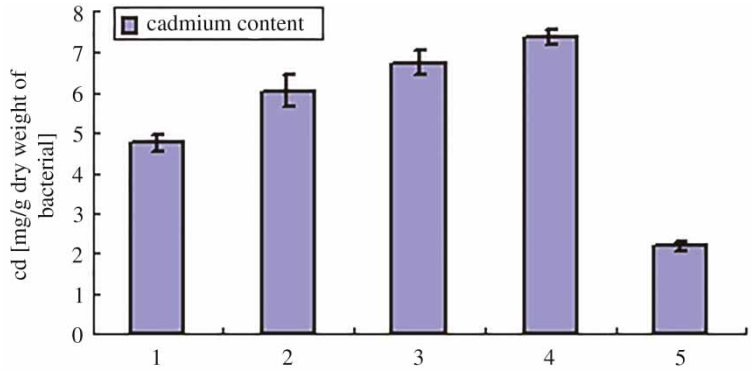

Figure 4 | Cadmium content in different *E. coli* BL21 strains. 1–5 represent the bacterium strains TP, AEP, ABEP, ABET3P and ET28a, respectively.

ordered from lowest to highest as follows: ET28a, TP, AEP, ABEP, and ABET3P. The cellular Cd content of the ET28a strain was significantly lower than that of the other strains. The Cd content in the ABET3P cells was approximately 3.5-fold greater than that measured in ET28a.

Wawrzynska *et al*. transformed the yeast gene SpPCS and the *E. coli* genes cysE and gshA into *E. coli* (Wawrzynska *et al*. 2005). Ideally, a high concentration of phytochelatins should be produced in the engineered bacteria. The production of phytochelatins is catalyzed by phytochelatin synthase, which is encoded by SpPCS. In addition, enough glutathione and cysteine should be produced as their production is catalyzed by r-glutamylcysteine synthase (encoded by the gshA gene) and serine acetyltransferase (encoded by the cysE gene) (Wawrzynska *et al*. 2005). However, the Cd resistance of the resulting bacterium did not improve (Wawrzynska *et al*. 2005). This result means that the limiting step for synthesizing glutathione was step 1, rather than step 2. If gshB could be transformed into *E. coli* with PCS, cysE, and gshA, the Cd resistance of the transformed bacterium could considerably increase. In the medium supplemented with 0.5 mmol/L $CdCl_2$, our results indicated that the OD_{600} values of the strains increased as follows: TP, AEP, ABEP, ET28a, and ABET3P. This result indicated that the glutathione content potentially decreased transiently, the metabolic processes related to glutathione were affected, and growth was inhibited after phytochelatins were over-produced. In contrast, after gshB was over-expressed, more glutathione was produced. The glutathione-related metabolic processes were recovered, and bacterial growth was promoted. The ABEP strain, in which gshA, gshB, cysE, and TcPCS1 were over-expressed, accumulated more cadmium (6.7 mg Cd/g dry weight of bacteria) than the AEP strain (6.1 mg Cd/g dry weight of bacteria). In the AEP strain, only gshA, cysE, and TcPCS1 were over-expressed (Figure 4), indicating that gshB can coordinate with gshA and cysE in recombinant *E. coli*. The strains that over-expressed gshB, gshA, and cysE could produce glutathione more efficiently than those that only expressed gshA and cysE.

T. caerulescens is well known for its ability to accumulate high levels of Cd (Milner & Kochian 2008). Some

ecotypes of *T. caerulescens* can accumulate Cd concentrations of up to 10,000 μg/g in the shoots. However, Cd concentrations greater than 1–10 μg/g are usually toxic in plants (Lombi *et al.* 2000). Furthermore, TcPCS1 is a homologous sequence for phytochelatins that is isolated from *T. caerulescens*. The AEP strain, which over-expresses gshA, cysE, and TcPCS1, contained 6.1 mg Cd/g dry weight of bacteria, which is significantly higher than that in the bacterium that over-expressed gshA, cysE, and SpPCS1 (Wawrzynska *et al.* 2005). This finding demonstrated that TcPCS1 is more specific and has a greater ability for detoxifying Cd than SpPCS1. In addition, HMA3 is a vacuolar heavy metal ATPase (Becher *et al.* 2004), and TcHMA3 is a determinant gene for Cd hyperaccumulation in *T. caerulescens* (Mendoza-Cozatl *et al.* 2011). The ABET3P, in which TcHMA3 was expressed, contained more Cd (7.5 mg Cd/g dry weight of bacteria) than the control ABEP (6.7 mg Cd/g dry weight of bacteria) (Figure 4), suggesting that TcHMA3 can facilitate Cd uptake not only in *T. caerulescens* but also in *E. coli*.

Conventional treatments are only effective when heavy metal ions are present at high concentrations (Ahluwalia & Goyal 2007). These treatments are often ineffective when Cd ions are present at micromolar concentrations (Colella *et al.* 2012). In contrast, engineered bacteria can still remove Cd under such conditions (Sauge-Merle *et al.* 2003). In practice, bioremediation using microorganisms is an effective and sustainable technology (Hashim *et al.* 2011). However, because Cd is also toxic to microorganisms, bacteria die when Cd concentrations are high. To overcome this problem, an engineered bacterium was developed in this study by transforming several exogenous genes. Phytochelatins can chelate Cd ions (Hirata *et al.* 2005). The toxicity of chelated Cd to bacterial cells is lower than that of Cd ions. The over-expression of TcPCS1 resulted in the ABET3P engineered bacterium, which contained more phytochelatins than the original bacterium. In addition, the ABET3P bacterium had more phytochelatins for chelation with Cd ions. The Cd tolerance of the engineered bacterium was greater than the original bacterium. The over-expression of gshA, gshB, and cysE endowed the engineered bacterium with the ability to produce more glutathione, which is used for synthesizing phytochelatins. If only TcPCS1 is over-expressed, the bacterium's Cd-tolerance could not be improved, despite the production of more phytochelatins, because the glutamine concentration fell short of the demand. In this case, the metabolic pathways related to glutamine were heavily affected. The over-expression of HMA3 confers the engineered bacterium the ability to accumulate more Cd than the control. The engineered bacterium ABET3P can function not only at millimolar concentrations of Cd but also at micromolar concentrations of Cd. ABET3P can accumulate significantly higher concentrations of Cd than the control. Thus, ABET3P has good potential for use in real wastewater.

CONCLUSIONS

In this study, an engineered bacterium strain, ABET3P, was developed. The engineered bacterium's resistance to Cd was significantly higher than that of the initial bacterium. In addition, the Cd accumulation in the engineered bacterium was much higher than that in the initial bacterium. This is the first engineered bacterial strain that has been reported with an improvement of not only Cd accumulation but also Cd tolerance. Thus, this bacterial strain has good potential for future use in wastewater treatment.

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