

# An operon consisting of a P-type ATPase gene and a transcriptional regular gene given the different cadmium resistances in *Bacillus vietamensis* 151-6 and *Bacillus marisflavi* 151-25

**Xiaoxia Yu**

Chinese academy of agricultural science

**Zundan Ding**

Chinese academy of agricultural science

**Yangyang Ji**

Jinan University

**Jintong Zhao**

Chinese Academy of Agricultural Sciences

**Xiaoqing Liu**

chinese academy of agricultural science

**Jian Tian**

chinese academy of agricultural science

**Ningfeng Wu** (✉ [wuningfeng@caas.cn](mailto:wuningfeng@caas.cn))

Biotechnology research institution, Chinese academy of agricultural science <https://orcid.org/0000-0002-7782-9920>

**Yunliu Fan**

chinese academy of agricultural science

---

## Research article

**Keywords:** Cd<sup>2+</sup> resistance; Genomic sequencing; *Bacillus vietamensis* 151-6; *Bacillus marisflavi* 151-25

**Posted Date:** August 3rd, 2019

**DOI:** <https://doi.org/10.21203/rs.2.11501/v1>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

**Version of Record:** A version of this preprint was published on January 21st, 2020. See the published version at <https://doi.org/10.1186/s12866-020-1705-2>.

# Abstract

Cadmium (Cd) is a severely toxic heavy metal to most microorganisms. Many bacteria have developed Cd<sup>2+</sup> resistance. In this study, we isolated two different Cd<sup>2+</sup> resistance *Bacillus* sp. strains, *Bacillus vietamensis* 151-6 and *Bacillus marisflavi* 151-25, which could be grown in the presence of Cd<sup>2+</sup> at concentration up to 0.3 mM and 0.8 mM, respectively. According to the genomic sequencing, transcriptome under cadmium stress and related biological experiments, a gene cluster in plasmid p25 containing orf4802 and orf4803, which encode an ATPase transporter and a transcriptional regulator protein, respectively, was a major contributor to Cd<sup>2+</sup> resistance in *B. marisflavi* 151-25. Although 151-6 has much lower Cd<sup>2+</sup> resistance than 151-25, they contain similar gene cluster, but in different locations. A gene cluster on the chromosome containing orf4111, orf4112 and orf4113, which encode an ATPase transporter, a cadmium efflux system accessory protein and a cadmium resistance protein, respectively, was found to be a major role on the Cd<sup>2+</sup> resistance for *B. vietamensis* 151-6. Based on homologies to the cad system (CadA-CadC) in *Staphylococcus aureus*, the mechanisms of cadmium resistance in *B. vietamensis* 151-6 and *B. marisflavi* 151-25 were as same as the cad system. Our study on the cadmium mechanism for *B. vietamensis* 151-6 and *B. marisflavi* 151-25 proved preliminarily that the cad system is widespread in *Bacillus* sp. bacteria.

## Introduction

Soil contamination with heavy metals is becoming an increasingly urgent problem worldwide. Among all heavy metals, one of the most hazardous is cadmium (Cd) (Abdu et al. 2016; Liu et al. 2016). Cd is a non-essential heavy metal element that is toxic, teratogenic and carcinogenic to humans. When critical Cd levels in soil are reached, biodiversity, agricultural productivity, food safety and human health can be threatened, as Cd can be accumulated in the food chain (Feng et al. 2018; Lehembre et al. 2013; Op De Beeck et al. 2015; Rehman et al. 2018; Satarug et al. 2003). Consequently, solutions to remediate heavy metal-contaminated soil, in particular Cd-contaminated soil, are urgently needed. Many remediation techniques, such as chemical immobilization, electrokinetic extraction, phytoremediation, and bioremediation, have been proposed for soils contaminated with heavy metals (Liu et al. 2018). Among these technologies, bioremediation is considered an innovative and promising remediation method based on its cost effectiveness and low environmental impact (Emenike et al. 2018; Jacob et al. 2018; Peng et al. 2018). To use microorganisms to remediate heavy metal contamination, in particular Cd-contaminated soil, it is first necessary to isolate bacteria that are resistant to Cd.

Many organisms have developed strategies to withstand the presence of Cd in the environment, such as exclusion, compartmentalization, deployment of inorganic polyphosphates, cell wall binding, and expression of metal binding proteins (Abbas et al. 2018; Gillan 2016; Kulakovskaya 2018; Reddy et al. 2016; Yu et al. 2018). The most prominent mechanism of resistance to Cd<sup>2+</sup> is the use of efflux pumps (Bruins et al. 2000). Several bacteria, such as *Alcaligenes eutrophus* (Nies et al. 1989), *Staphylococcus aureus* (Nucifora et al. 1989), *Bacillus subtilis* (Moore et al. 2005), *Listeria monocytogenes* (Parsons et al. 2017) and *Escherichia coli* (Khan et al. 2015) have been demonstrated resistance to Cd<sup>2+</sup>.

One of the most thoroughly characterized Cd<sup>2+</sup> resistance efflux systems is the *czc* (Cd<sup>2+</sup>, Zn<sup>2+</sup>, and Co<sup>2+</sup> resistance) system in the gram-negative bacterium *Alcaligenes eutrophus* (Diels et al. 1995; Nies et al. 1989). The CzcA, CzcB and CzcC proteins comprise an active efflux pump complex driven by a cation-proton antiporter (Nies et al. 1989). Briefly, CzcA, located in the inner membrane, is essential for cation transport and can remove heavy metals (Cd<sup>2+</sup>, Zn<sup>2+</sup>, and Co<sup>2+</sup>) from the cytoplasm using an H<sup>+</sup> ion gradient (Diels et al. 1995; Nies 1992). CzcB belongs to a family of bacterial membrane fusion proteins. It may create a pathway for the removal of cations from CzcA (Diels et al. 1995; Rensing et al. 1997b). CzcC relies on CzcB to function and may act as a substrate (Cd<sup>2+</sup>, Zn<sup>2+</sup>, and Co<sup>2+</sup>) switch for the efflux pump (Bruins et al. 2000).

Another well-characterized Cd<sup>2+</sup> resistance system is the *cad* system(*cadA-cadC*) in the gram-positive bacterium *S. aureus* (Nucifora et al. 1989; Yoon and Silver 1991). The Cd-efflux ATPase is encoded by the *cadA* gene, which contains six predicted membrane-spanning regions. The fourth membrane span is thought to be involved in the cation translocation pathway, and includes a conserved Cys-Pro-Cys tripeptide (Silver and Phung 1996). CadC is a regulatory protein encoded immediately downstream of *cadA*, and is also required for Cd<sup>2+</sup> resistance in *S. aureus* (Yoon et al. 1991). CadC is a member of the ArsR/SmtB family (Busenlehner et al. 2003; Saha et al. 2017), which can bind to the promoter-operator area of the *cadA-cadC* system and acts as a transcriptional repressor in vitro (Endo and Silver 1995).

In present study, two different Cd<sup>2+</sup> resistance *Bacillus* sp. strains(*B. vietamensis* 151-6 and *B. marisflavi* 151-25) isolated from Cd-contaminated soil was grown in the presence of Cd<sup>2+</sup> at two distinct concentrations up to 0.3 mM and 0.8 mM, respectively. In order to compare the mechanism of Cd<sup>2+</sup> resistance for these two strains, their genome and transcriptome under Cd<sup>2+</sup> stress were analyzed. Moreover, a fosmid library from genomic DNA of *B. vietamensis* 151-6 was constructed to further delineate the Cd<sup>2+</sup> resistance-related genes. The functions of these genes were deeply analyzed and verified through overexpression in *E. coli* and *B. subtilis*. The gene clusters of 4802-4803 in the plasmid p25 of *B. marisflavi* 151-25 and 4111-4112-4113 on the chromosome of *B. vietamensis* 151-6 played the major role on the Cd<sup>2+</sup> resistance.

## Materials And Methods

### 2.1 Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table S1. *B. subtilis* WB600 (BS), *B. amyloliquefaciens* (BA) and *B. licheniformis* WX-02 (BL) were stored in our lab. *E. coli* TOP10 (TIANGEN Biotech, Beijing, China) was used as the cloning host. *B. vietamensis* 151-6 and *B. marisflavi* 151-25 were isolated from Cd-contaminated soil in Hunan Province (27°46'N, 112°52'E), China. These strains were all grown in Luria–Bertani (LB) medium. CdCl<sub>2</sub>·H<sub>2</sub>O was used as the source of Cd. Ampicillin (100 µg/mL), kanamycin (10 µg/mL), or tetracycline(5 µg/mL) was added as necessary.

## 2.2 Isolation of Cd-resistant bacteria

Cd-contaminated soil was collected from a former industrial site in Hunan Province (27°46'N, 112°52'E) and analyzed for Cd content through acid digestion followed by the use of a 7,700 × inductively coupled plasma mass spectrometer (Agilent Technologies, Tokyo, Japan). To isolate Cd-resistant and safe bacteria, aerobic *Bacillus* sp. were isolated from the soil by plating on LB agar plates containing progressively higher concentrations of cadmium chloride (0, 0.5, 1 and 2 mM) after bacterial enrichment cultures were heat-shocked at 80°C for 20 min. Bacterial genomic DNA was isolated using the TIANGEN Bacteria DNA kit (TIANGEN Biotech). The 16S rRNA gene was amplified from the extracted DNA using the universal primers 16S rRNA-F/16S rRNA-R (Table S2) and the amplification products were cloned in the pGM-T (TIANGEN Biotech) vector using competent *E. coli* TOP10 cells (TIANGEN Biotech). Sequencing was carried out using T7 and SP6 primers and compared to the GenBank database using the NCBI BLAST program.

### Evaluation of cadmium resistance and growth curve

To evaluate growth in a liquid medium, the minimum inhibitory concentration (MIC) of Cd<sup>2+</sup> (MIC-Cd) was determined. LB medium (800 µL) with different concentrations of Cd<sup>2+</sup> was dispensed into 96-well (12 × 8) microtiter plates (96 × 2-mL wells) with a multi-channel micropipette. Single colonies of the test strains were inoculated into 3 mL of LB medium and cultured overnight. The test culture (15 µL) was then inoculated into each well of the prepared 96-well plate. After 24 h at 37°C and 750 rpm in an incubator (Heidolph, Viertrieb, Germany), 200 µL of the cell suspension was transferred to a 96-well plate and the turbidity at OD<sub>600</sub> was measured.

To determine the tolerance to Cd<sup>2+</sup> of bacteria, growth curves at different concentrations of Cd<sup>2+</sup> were analyzed. For the growth assay, single colonies of the test strains were cultured overnight and then diluted 1:100 into 100-well plates containing 200 µL of LB and various concentrations of Cd<sup>2+</sup> in quintuplicate. The growth curve was measured at 1-h intervals using a Bioscreen C automatic growth curve analyzer (Bioscreen, Helsinki, Finland).

### Genome sequencing and analysis

Bacterial genomic DNA was extracted using the sodium dodecyl sulfate (SDS) method (Lee et al. 2003). A total of 5 µg DNA was used to generate each library, and this DNA was sheared using Covaris g-Tubes to generate sheared fragments >10 kb in length. The sheared DNA fragments were then prepared using the SMRT bell template preparation kit (Pacific Biosciences, Menlo Park, CA, USA) according to the manufacturer's instructions. Whole-genome sequencing was performed on the Pacbio RSII platform. All high-quality paired reads were assembled using SOAPdenovo (<http://soap.genomics.org.cn/soapdenovo.html>) onto a number of scaffolds (Li et al. 2010). Then, the filtered reads were transferred for the next step of gap closing. Transfer RNA (tRNA) genes were predicted with tRNAscan-SE (Lowe and Eddy 1997). Ribosomal RNA (rRNA) genes were analyzed using rRNAmmer

(Lagesen et al. 2007). Coding genes were identified with the GeneMarkS program (Besemer et al. 2001). The predicted coding genes were annotated based on the non-redundant protein database (NR) of the National Center for Biotechnology Information (Li et al. 2002) and Gene Ontology (GO) (Ashburner et al. 2000).

### RNA sequencing and transcriptome analysis

An overnight culture was diluted 1:100 in LB medium in the presence (0.1 mM) and absence of Cd<sup>2+</sup>, and these cultures were grown at 37°C and 200 rpm to the exponential phase. Total RNA from both groups (each group has three replicates) were extracted using a TRIzol kit (TIANGEN Biotech) according to the manufacturer's instructions. A total of 1 µg RNA per sample was used as input material for RNA sample preparation. Sequence libraries were generated using the Illumina® TruSeq® Stranded Total RNA Sample Preparation kit (NEB, Ipswich, MA, USA). TruSeq Stranded Total RNA was prepared with the Ribo-Zero™ Bacteria Kit (Epicenter, Madison, WI, USA). The clustering of index-coded samples was performed on a cBot Cluster Generation System using the TruSeq HiSeq 4000 PE 150 Cluster Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. After cluster generation, the prepared libraries were sequenced on the Illumina HiSeq 4000 platform by Allwegene Technology (Beijing, China) and 150-bp paired-end reads were generated. Bowtie2 was used to map mRNA reads to the genome, and HTSeq v 0.5.4 p3 was used to count the number of reads mapped to each gene. Then, the fragments per kilobase of exon model per million mapped reads (FPKM) of each gene was calculated based on the length of, and read count mapped to, the gene (Mortazavi et al. 2008).

### Elimination of plasmids

Plasmid elimination was performed using SDS, as previously described (El-Mansi et al. 2000), with some modifications. Specifically, *B. vietnamsis* 151-6 and *B. marisflavi* 151-25 were treated with 0.0005% and 0.002% SDS, respectively. Single colonies were selected on LB agar plates to screen for strains that had eliminated the p6 plasmid or the p25 plasmid. Primers 4108CDS-F/4108CDS-R and 4109CDS-F/4109CDS-R (Table S2) were used to identify gene located on the chromosome of *B. vietnamsis* 151-6. The primer pairs 4963CDS-F/4963-CDS-R, 4967CDS-F/4967CDS-R, 4982CDS-F/4982CDS-R, 4983CDS-F/4983CDS-R, 5014CDS-F/5014CDS-R and 5018CDS-F/5018CDS-R (Table S2) were employed to identify the genes in the plasmid p6 (Fig. S6) For *B. marisflavi* 151-25, the primers 4163CDS-F and 4163CDS-R (Table S2) were used to identify genes located on the chromosome, while the primer pairs 4779CDS-F/4779-CDS-R, 4780CDS-F/4780CDS-R and 4803CDS-F/4803CDS-R (Table S2) were used to detect the p25 plasmid (Fig. S7). Meanwhile, the primers for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Table S2) located on plasmid p25 were also used to detect the plasmid (Fig. S7).

### RT-PCR and qRT-PCR

Total RNA of *B. vietnamsis* 151-6 and *B. marisflavi* 151-25 were isolated as described above under RNA sequencing. cDNA samples were prepared using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) with random primers. qRT-PCR was performed

using the ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech, Beijing, China) according to the manufacturer's instructions. 16S rRNA, amplified with the primers 16S-F/16S-R, was used as an internal control. All reactions were performed in biological triplicate, and the normalized fold changes of the relative expression ratios were quantified using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen 2001).

#### Construction and screening of an *B. vietnamsis* 151-6 genomic fosmid library

High-molecular-weight genomic DNA from *B. vietnamsis* 151-6 was extracted using the bacterial genomic DNA extraction kit (BioTeke Biotech, Beijing, China) according to the manufacturer's instructions. And a fosmid library was constructed with the CopyControl Fosmid Library Production Kit (vector pCC1FOS; Epicentre, Madison, WI, USA) according to the manufacturer's instructions. A total of 1204 clones were used to construct the library in 13 96-well plates. To screen for clones with Cd<sup>2+</sup> resistance, overnight cultures of the 1204 clones were inoculated into LB liquid medium with 1.0 mM and 1.2 mM Cd<sup>2+</sup>, respectively. Meanwhile, these clones were spotted onto LB agar plates with 1.2 mM and 1.5 mM Cd<sup>2+</sup>. Fosmid DNA was isolated from the positive fosmid clones by Axyprep Plasmid Miniprep Kit (Axygen, USA) and sequenced by pCC1 sequencing primers pCC1-F/ pCC1-R (Table S2). The results were aligned with the genome sequence of *B. vietnamsis* 151-6 to confirm the sequence of the inserted fragments.

#### Construction of plasmids for gene fragments heterologous expression

To verify that Cd<sup>2+</sup> resistance is conferred by genes, recombinant pUC19 and pUBC19 plasmids were constructed. The 3444-bp *Sac*I-*Xba*I DNA fragment amplified from *B. vietnamsis* 151-6 genome with the primers 4111-4112-4113-F/4111-4112-4113-R (Table S2), containing copper-translocating P-type ATPase gene (*orf4111*), cadmium efflux system accessory gene *cadC* (*orf4112*), cadmium resistance gene *cadD* (*orf4113*) and 217-bp upstream of *orf4113*, was cloned into *E. coli* pUC19 vector and *E. coli*-*B. subtilis* shuttle vector pUBC19, respectively. The gene fragments 4087-4088, 4093-4094-4095, 4102-4103 and 4108-4109 amplified from *B. vietnamsis* 151-6 genome with corresponding primers (Table S2) were also cloned into pUC19 and pUBC19. For *B. marisflavi* 151-25, the gene fragments 4774-4775, 4776-4777, 4779-4780, 4781-4782 and 4802-4803 amplified from plasmid p25 of *B. marisflavi* 151-25 with the corresponding primers (Table S2) were cloned into pUC19 and pUBC19. The ligation mixture was transformed into *E. coli* TOP10, and the correct plasmids were identified through colony PCR with corresponding sequencing primers (Table S2). After confirmation via sequencing, recombinant pUBC19 plasmids were extracted from *E. coli* TOP10 and transformed into *B. subtilis*, as described previously (Yu, et al. 2015). Transformants were harvested by screening the clones on LB agar containing 10 µg/mL kanamycin, and the correct plasmids were verified through colony PCR with the pUBC19 sequencing primers CX-F/CX-R (Table S2).

#### MIC-Cd and growth curve determination

MIC-Cd and the growth curve of plasmid-eliminated strains and wild strains of *B. vietnamsis* 151-6 and *B. marisflavi* 151-25 were determined as described above in 2.3. MIC-Cd values for the recombinant strains of *E. coli* TOP10 and *B. subtilis* were also evaluated using 96-well microtiter plates, as described in 2.3.

Data availability

The *B. vietnamsis* 151-6 chromosome and plasmid p6, also the chromosomal and p25 plasmid sequences of *B. marisflavi* 151-25 have been deposited in GenBank under accession number SUB5733407 and SUB4501425.

## Results

### 3.1 Strain 151-6 and 151-25 exhibit different Cd<sup>2+</sup> resistances

To isolate Cd-resistant bacteria, a Cd-contaminated ( $59.561 \pm 3.76$  mg Cd kg<sup>-1</sup>) soil sample was collected in Hunan Province. A total of 21 strains of bacteria were isolated using LB agar plates containing 0.5, 1 and 2 mM Cd<sup>2+</sup>. The MIC-Cd values of those isolates were determined and ranged from 0.4 to 1.0 mM (Table S3). The strains of 151-25 and 151-6, which showed the highest and lowest level of Cd<sup>2+</sup> resistance respectively, were selected for further study (Table S3), and were identified as *Bacillus* sp. through 16S rDNA analysis.

To compare the level of Cd<sup>2+</sup> resistance of two selected strains with other *Bacillus* sp. bacteria, we examined their MIC-Cd, as well as those of three other *Bacillus* sp. strains, BS, BA and BL, in liquid medium supplemented with various Cd<sup>2+</sup> concentrations. 151-25 and 151-6 were grown in the presence of Cd<sup>2+</sup> at concentrations up to 0.8 mM and 0.3 mM, respectively (Fig. 1a), while the growth of the other three *Bacillus* sp. strains was inhibited in the presence of 0.3 mM Cd<sup>2+</sup> (Fig. 1a). The growth curves at 0 mM Cd<sup>2+</sup> demonstrated that all four *Bacillus* sp. strains exhibited similar growth trends in liquid LB medium (Fig. S1a). However, under culture conditions with 0.1 mM or 0.3 mM Cd<sup>2+</sup>, 151-6 and 151-25 exhibited greater growth potential than the other three *Bacillus* sp. strains (Fig. S1b and Fig. 1c). And only 151-25 could grow at 0.5 mM Cd<sup>2+</sup> (Fig. S1d). These data indicated that compared to other *Bacillus* sp. strains, 151-6 and 151-25 exhibited stronger tolerance to Cd<sup>2+</sup>. And the Cd<sup>2+</sup> resistance of 151-25 was significantly higher than 151-6.

### 3.2 Genomic analysis of 151-6 and 151-25

To compare the mechanism of Cd<sup>2+</sup> resistance for 151-6 and 151-25, their genome were sequenced. The results showed that 151-6 contains a single chromosome (4,556,861bp and 4,952 predicted genes) and one plasmid, designated p6 (40,946bp and 66 predicted genes) (Fig. S2). And 151-25 also contains a

single chromosome (4,411,234 bp and 4,734 predicted genes) and one plasmid, designated p25 (138,020 bp and 156 predicted genes) (Fig. S3). Simultaneously, the results of non-redundant protein database (NR) annotation of the predicted coding genes presented that strain 151-6 can be identified as *B. vietnamensis* (4093 of the 5018 genes are annotated to derive from *B. vietnamensis*) and strain 151-25 can be identified as *B. marisflavi* (4095 of the 4890 genes are annotated to derive from *B. vietnamensis*).

3.3 Plasmid p25 in *B. marisflavi* 151-25 is important for Cd<sup>2+</sup> resistance, while p6 in *B. vietnamensis* 151-6 is not relevant to the Cd<sup>2+</sup> resistance

To determine whether plasmid p6 and p25 were responsible for Cd<sup>2+</sup> resistance in *B. vietnamensis* 151-6 and *B. marisflavi* 151-25, we eliminated the plasmids and then examined their contribution to Cd<sup>2+</sup> resistance. The plasmid p6-eliminated strains 151-6 $\Delta$ 5 and 151-6 $\Delta$ 7 and the plasmid p25-deleted strains 151-25 $\Delta$ 3 and 151-25 $\Delta$ 29 were obtained (Fig. S6a and Fig. S7a, respectively) and further verified by PCR (Fig. S6b and Fig. S7b, 7c, respectively). In liquid media, the strains 151-6 $\Delta$ 5 and 151-6 $\Delta$ 7 exhibited the same MIC-Cd as the wild strain 151-6 (Fig. 2a). And the growth curve of 151-6 $\Delta$ 5 and 151-6 $\Delta$ 7 did not show the difference with wild type 151-6 at the three different concentrations of Cd<sup>2+</sup> (Fig. 2b, 2c and 2d). These data indicated that the elimination of plasmid p6 does not influence the Cd<sup>2+</sup> resistance of *B. vietnamensis* 151-6. However, eliminating plasmid p25 made a significantly decreased of Cd<sup>2+</sup> resistance in *B. marisflavi* 151-25. As shown in Fig. 3a, the MIC-Cd values of 151-25 $\Delta$ 3 and 151-25 $\Delta$ 29 were 0.2 mM, markedly lower than that of wild-type 151-25 (1.0 mM). Elimination of plasmid p25 did not affect the growth of the strain in liquid LB medium without added Cd<sup>2+</sup> (Fig. 3b). However, with addition of 0.1 or 0.5 mM Cd<sup>2+</sup> to the medium, the growth of 151-25 $\Delta$ 3 was inhibited, while the growth of *B. marisflavi* 151-25 was not affected (Fig. 3c and 3d, respectively). Thus, we concluded that plasmid p25 is important for Cd<sup>2+</sup> resistance in *B. marisflavi* 151-25 while plasmid p6 is not relevant to the Cd<sup>2+</sup> resistance in *B. vietnamensis* 151-6.

### 3.4 A gene cluster consisting of *orf4802* and *orf4803* confers high Cd<sup>2+</sup> resistance in *B. marisflavi* 151-25

To determine the genes related to the Cd<sup>2+</sup> resistance of *B. marisflavi* 151-25, its transcriptome under Cd<sup>2+</sup> induction was analyzed by RNA sequencing. Fragments per kilobase of exon model per million mapped reads values were used to measure transcript abundance. As shown in Table S4 and Fig. S5, a total of 65 differentially expressed genes were identified in the presence of Cd<sup>2+</sup> (47 up-regulated genes and 18 down-regulated genes). Among the 47 up-regulated genes, 9 were located on plasmid p25, and the top three up-regulated genes in terms of fold change were also from plasmid p25 (Tables 1 and S4). Briefly, transcripts of a transcriptional regulator gene (*orf4803*) and a copper-translocating P-type ATPase gene (*orf4802*) were increased 72.53- and 63.83-fold, respectively. These results were confirmed through qRT-PCR (Fig. 4b), and it further indicated that plasmid p25 is related to Cd<sup>2+</sup> resistance in *B. marisflavi* 151-25. Based on these results, we hypothesized that nine up-regulated genes located on plasmid p25,

*orf4774*, *orf4775*, *orf4776*, *orf4777*, *orf4779*, *orf4781*, *orf4782*, *orf4802* and *orf4803* may be involved in Cd<sup>2+</sup> resistance of *B. marisflavi* 151-25. To confirm this hypothesis, we overexpressed these genes using their own promoter with five gene clusters (Fig. 4a) in *E. coli* and *B. subtilis*, to examine their contributions to Cd<sup>2+</sup> resistance. As shown in Fig. 4c and 4d, the gene cluster containing *orf4802* and *orf4803* allowed recombinant *E. coli* and *B. subtilis* to exhibit higher Cd<sup>2+</sup> resistance than negative control strains. In particular, *B. subtilis* cells containing the recombinant plasmid 4802-4803-pUBC19 would grow in the presence of Cd<sup>2+</sup> at seven times the concentration tolerated by control cells. However, compared to control cells, the other four recombinant *E. coli* and *B. subtilis* strains did not show elevated Cd<sup>2+</sup> resistance. These results indicate that the gene cluster consisting of *orf4802* and *orf4803* confers high Cd<sup>2+</sup> resistance in *B. marisflavi* 151-25.

### **3.5 A gene cluster consisting of *orf4111*, *orf4112* and *orf4113* confers Cd<sup>2+</sup> resistance for *B. vietnamsis* 151-6**

Compared to *B. marisflavi* 151-25, the analysis of the transcriptome of *B. vietnamsis* 151-6 under Cd<sup>2+</sup> induction demonstrated that a total of 1268 differentially expressed genes were identified (585 up-regulated genes and 683 down-regulated genes), significantly higher than that of *B. marisflavi* 151-25 (Fig. S4 and Table S5). Moreover, 585 up-regulated genes in the presence in Cd<sup>2+</sup> were located on the chromosome, and among the 683 down-regulated genes, only 16 were located on plasmid p6. These data verified that plasmid p6 does not influence the Cd<sup>2+</sup> resistance of *B. vietnamsis* 151-6. To further identify the key Cd<sup>2+</sup> resistance genes from those differentially expressed genes, a fosmid library of *B. vietnamsis* 151-6 genomic DNA was constructed in *E. coli*. And the clones were screened by the Cd<sup>2+</sup> resistance to determine the genes that contribute to Cd<sup>2+</sup> resistance. Among the total of 1204 clones, there were 25 and 3 clones could be grown on LB agar plates with 1.2 mM and 1.5 mM Cd<sup>2+</sup>, respectively. In liquid LB medium with 1.2 mM Cd<sup>2+</sup>, there were 27 clones could be grown. Only 2 clones, B2 and C2, could be grown in liquid LB medium with 1.5 mM Cd<sup>2+</sup>. The MIC-Cd of B2 and C2 were determined. As shown in Fig. S8, B2 and C2 exhibit higher Cd<sup>2+</sup> resistance than negative control strain EPI300-T1<sup>R</sup>. The fosmid DNAs isolated from B2 and C2 were sequenced, respectively. Sequence analyses of B2 revealed that it contained 32,263bp insert fragment, which consisted 31 annotated genes (Table 2) (And the fosmid DNA isolated from C2 was failed by sequencing many times, so the clone was not analyzed). Moreover, the analysis of the transcriptome showed that insert fragment of B2 contained 8 up-regulated genes (*orf4108*, *orf4109*, *orf4088*, *orf4087*, *orf4090*, *orf4093*, *orf4106*, *orf4107*) and 3 down-regulated genes (*orf4086*, *orf4101*, *orf4120*). These results were confirmed through qRT-PCR and the transcription levels of other 16 genes in the insert fragment of B2 were also evaluated by qRT-PCR. The results presented that the transcripts of the *orf4108*, *orf4109*, *orf4088*, *orf4087*, *orf4104*, *orf4106* and *orf4107* were increased 156.64, 130.84, 102.58, 87.26, 14.19, 12.68 and 9.07-fold, respectively (Table 2 and Fig. 5b). Based on these results, four gene clusters (*4108-4109*, *4087-4088*, *4104*, *4106-4107*) containing their own promoter were overexpressed in *E. coli* and *B. subtilis* to examine their contributions to the Cd<sup>2+</sup>

resistance. Due to the fact that ATPase gene and oxidoreductase gene have been reported to involved in Cd<sup>2+</sup> resistance (Wang and Crowley 2005), three related gene clusters (4093-4094-4095, 4102-4103 and 4111-4112-4113) containing their own promoter were also overexpressed in *E. coli* and *B. subtilis* (And the construction of the recombinant vectors containing 4104 and 4106-4107 were failed by many times, so the results were not showed). As shown in Fig. 5c and Fig. 5d, the gene cluster containing *orf4111* (copper-translocating P-type ATPase gene), *orf4112* (cadmium efflux system accessory protein gene, *cadC*) and *orf4113* (cadmium resistance protein gene, *cadD*) allowed recombinant *E. coli* and *B. subtilis* to exhibit higher Cd<sup>2+</sup> resistance than the negative control strain. Especially, the *B. subtilis* cells containing the recombinant plasmid 4111-4112-4113-pUBC19 demonstrated the potential to tolerate and grow in the presence of 6 times the concentration of Cd<sup>2+</sup> compared to the control cells. Moreover, the recombinant strain exhibited similar Cd resistance with the strain which overexpressed the gene fragment 4802-4803 from *B. marisflavi* 151-25 (Fig. 4d). However, compared to the negative control cells, the other 4 recombinant *E. coli* and *B. subtilis* cells did not show great Cd<sup>2+</sup> resistance. These results indicated that the gene cluster consisting of *orf4111*, *orf4112* and *orf4113* confers Cd<sup>2+</sup> resistance for *B. vietnamsis* 151-6.

## Discussion

The present study showed that the gene cluster 4802-4803 located on plasmid p25 and the gene cluster 4111-4112-4113 located on chromosome were involved in Cd<sup>2+</sup> resistance for strain *B. marisflavi* 151-25 and *B. vietnamsis* 151-6, respectively. Many microorganisms have been reported to use heavy-metal-transporting ATPases, such as the proteins CadA and ZntA, to overcome Cd<sup>2+</sup> toxicity (Nucifora et al. 1989; Rensing, et al. 1997a). Specifically, CadA from *S. aureus* has a length of 727 amino acids (Nucifora et al. 1989), and its amino acid sequence is highly similar to 4111 (63.31%) and 4802 (65.21%) (Fig. S9). Alignment with CadA from *S. aureus* showed that the protein sequences of *orf4111* from *B. vietnamsis* 151-6 and *orf4802* from *B. marisflavi* 151-25 all included a conserved (Cys-Pro-Cys) tripeptide for Cd<sup>2+</sup>, Pb<sup>2+</sup> or Zn<sup>2+</sup> binding (Ye et al. 2005). CadC from *S. aureus* including 122 amino acids was related to the divalent cation ATPase (Silver and Phung 1996). Expression of CadA is regulated by CadC, which is a homodimeric repressor that dissociates from the *cad* operator/promoter upon binding (Nucifora et al. 1989). CadC is a member of the ArsR/SmtB family of metalloregulatory proteins (Busenlehner et al. 2003; Saha, et al. 2017). Its crystal structure was resolved in 2005, and showed that two regulatory metal-binding sites for the inducer Cd<sup>2+</sup> are formed by Cys-7 and Cys-11 from the N terminus of one monomer and Cys-58 and Cys-60 of the other monomer (Ye et al. 2005). Alignment with CadC from *S. aureus* showed that the protein sequence of *orf4112* from *B. vietnamsis* 151-6 and of *orf4803* from *B. marisflavi* 151-25 contained these four Cys residues (Fig. S10). Moreover, the similarity between CadC and Orf4803 or Orf4112 are 86.89% and 82.64%, respectively. These analysis indicated that the Cd<sup>2+</sup> resistance mechanisms of *B. vietnamsis* 151-6 and *B. marisflavi* 151-25 were all the *cad* system, and a gene cluster consisting a P-type ATPase gene and a transcriptional regular gene given the different

cadmium resistances in *B. vietnamsis* 151-6 (4111-4112 located on chromosome) and *B. marisflavi* 151-25 (4802-4803 located on plasmid). The hypothetical *cad* model was showed in Fig. S11.

To find more genes responsible for high Cd<sup>2+</sup> resistance of *B. marisflavi* 151-25, we also compared gene expression between *B. marisflavi* 151-25 and 151-25 $\Delta$ 3 under Cd<sup>2+</sup> stress. 19 up-regulated genes located on the chromosome that exhibited a more than 4-fold change according to transcriptome data were analyzed using qRT-PCR. Compared to wild-type *B. marisflavi* 151-25, the transcript levels of seven genes showed marked fold increases in 151-25 $\Delta$ 3 (Fig. S12a). These genes were *orf666* (TetR family transcriptional regulator gene), *orf667* (cysteine ABC transporter substrate-binding protein gene), *orf668* (ABC transporter permease gene), *orf1240* (ArsR family transcriptional regulator gene), *orf1241* (copper-translocating P-type ATPase gene), *orf3892* (hypothetical protein gene) and *orf3894* (cation transporter gene). To confirm whether these genes related to Cd<sup>2+</sup> resistance for *B. marisflavi* 151-25, three gene fragments also with 4802-4803 were overexpressed in *B. subtilis* and the Cd-MIC values for those recombinant strains were determined. As shown in Fig. 12b, the operon containing *orf4802* and *orf4803* allowed recombinant *B. subtilis* to exhibit greater Cd<sup>2+</sup> resistance, while the other three fragments would not increase Cd<sup>2+</sup> resistance of the recombinant *B. subtilis*. Our results suggested that the operon of 4802-4803 plays a leading role for Cd<sup>2+</sup> resistance of *B. marisflavi* 151-25, and that when plasmid p25 (containing the main Cd-efflux pump genes, *orf4802* and *orf4803*) was deleted, the transcript level of other efflux pump genes would be enhanced. The interaction between these genes remains to be further study.

Overall, we identified that the Cd<sup>2+</sup> resistance mechanism of *B. vietnamsis* 151-6 and *B. marisflavi* 151-25 were all *cad* system. The *cad* system was also reported in *B. firmus* and *B. subtilis* (Ivey et al. 1992; Solovieva and Entian 2002), but not in *B. vietnamsis* and *B. marisflavi*. Our results further confirm that the *cad* system is widespread in *Bacillus* sp. bacteria.

## Declarations

## Acknowledgments

We would like to thank all colleagues in our lab for constructive discussion and technical support. This research was supported by funding from National Natural Science Foundation of China (NSFC, Grant No. 31770124) and Agricultural Science and Technology Innovation Program of Chinese Academy of Agricultural Sciences (Grant No. CAAS-XTCX2016018).

## Conflicts of interest

The authors declare that they have no conflicts of interest.

## References

Abbas SZ, Rafatullah M, Hossain K, Ismail N, Tajarudin HA, Khalil H (2018) A review on mechanism and future perspectives of cadmium-resistant bacteria. *International Journal of Environmental Science and Technology* 15: 243-262 doi: 10.1007/s13762-017-1400-5

Abdu N, Abdullahi AA, Abdulkadir A (2016) Heavy metals and soil microbes. *Environmental Chemistry Letters* 15: 65-84 doi: 10.1007/s10311-016-0587-x

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nature genetics* 25: 25-29 doi: 10.1038/75556

Besemer J, Lomsadze A, Borodovsky M (2001) GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions. *Nucleic Acids Res* 29: 2607-2618 doi:

Bruins MR, Kapil S, Oehme FW (2000) Microbial resistance to metals in the environment. *Ecotoxicol Environ Saf* 45: 198-207 doi: 10.1006/eesa.1999.1860

Busenlehner LS, Pennella MA, Giedroc DP (2003) The SmtB/ArsR family of metalloregulatory transcriptional repressors: Structural insights into prokaryotic metal resistance. *FEMS Microbiol Rev* 27: 131-143 doi:

Diels L, Dong Q, van der Lelie D, Baeyens W, Mergeay M (1995) The *czc* operon of *Alcaligenes eutrophus* CH34: from resistance mechanism to the removal of heavy metals. *J Ind Microbiol* 14: 142-153 doi:

El-Mansi M, Anderson KJ, Inche CA, Knowles LK, Platt DJ (2000) Isolation and curing of the *Klebsiella pneumoniae* large indigenous plasmid using sodium dodecyl sulphate. *Res Microbiol* 151: 201-208 doi:

Emenike CU, Jayanthi B, Agamuthu P, Fauziah SH (2018) Biotransformation and removal of heavy metals: a review of phytoremediation and microbial remediation assessment on contaminated soil. *Environmental Reviews* 26: 156-168 doi: 10.1139/er-2017-0045

Endo G, Silver S (1995) CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid pI258. *J Bacteriol* 177: 4437-4441 doi:

Feng G, Xie T, Wang X, Bai J, Tang L, Zhao H, Wei W, Wang M, Zhao Y (2018) Metagenomic analysis of microbial community and function involved in cd-contaminated soil. *BMC microbiology* 18: 11 doi: 10.1186/s12866-018-1152-5

Gillan DC (2016) Metal resistance systems in cultivated bacteria: are they found in complex communities? *Curr Opin Biotechnol* 38: 123-130 doi: 10.1016/j.copbio.2016.01.012

- Ivey DM, Guffanti AA, Shen Z, Kudyan N, Krulwich TA (1992) The cadC gene product of alkaliphilic *Bacillus firmus* OF4 partially restores Na<sup>+</sup> resistance to an Escherichia coli strain lacking an Na<sup>+</sup>/H<sup>+</sup> antiporter (NhaA). J Bacteriol 174: 4878-4884 doi: 10.1128/jb.174.15.4878-4884.1992
- Jacob JM, Karthik C, Saratale RG, Kumar SS, Prabakar D, Kadirvelu K, Pugazhendhi A (2018) Biological approaches to tackle heavy metal pollution: A survey of literature. Journal of environmental management 217: 56-70 doi: 10.1016/j.jenvman.2018.03.077
- Khan Z, Nisar MA, Hussain SZ, Arshad MN, Rehman A (2015) Cadmium resistance mechanism in *Escherichia coli* P4 and its potential use to bioremediate environmental cadmium. Applied microbiology and biotechnology 99: 10745-10757 doi: 10.1007/s00253-015-6901-x
- Kulakovskaya T (2018) Inorganic polyphosphates and heavy metal resistance in microorganisms. World J Microbiol Biotechnol 34:10.1007/s11274-018-2523-7
- Lagesen K, Hallin P, Rodland EA, Staerfeldt HH, Rognes T, Ussery DW (2007) RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res 35: 3100-3108 doi: 10.1093/nar/gkm160
- Lee YK, Kim HW, Liu CL, Lee HK (2003) A simple method for DNA extraction from marine bacteria that produce extracellular materials. J Microbiol Methods 52: 245-250 doi: 10.1016/s0167-7012(02)00180-x
- Lehembre F, Doillon D, David E, Perrotto S, Baude J, Foulon J, Harfouche L, Vallon L, Poulain J, Da Silva C, Wincker P, Oger-Desfeux C, Richaud P, Colpaert JV, Chalot M, Fraissinet-Tachet L, Blaudez D, Marmeisse R (2013) Soil metatranscriptomics for mining eukaryotic heavy metal resistance genes. Environmental Microbiology 15: 2829-2840 doi: doi:10.1111/1462-2920.12143
- Li R, Zhu H, Ruan J, Qian W, Fang X, Shi Z, Li Y, Li S, Shan G, Kristiansen K, Li S, Yang H, Wang J, Wang J (2010) De novo assembly of human genomes with massively parallel short read sequencing. Genome research 20: 265-272 doi: 10.1101/gr.097261.109
- Li W, Jaroszewski L, Godzik A (2002) Tolerating some redundancy significantly speeds up clustering of large protein databases. Bioinformatics (Oxford, England) 18: 77-82 doi:
- Liu L, Li W, Song W, Guo M (2018) Remediation techniques for heavy metal-contaminated soils: Principles and applicability. The Science of the total environment 633: 206-219 doi: 10.1016/j.scitotenv.2018.03.161
- Liu X, Tian G, Jiang D, Zhang C, Kong L (2016) Cadmium (Cd) distribution and contamination in Chinese paddy soils on national scale. Environmental science and pollution research international 23: 17941-17952 doi: 10.1007/s11356-016-6968-7
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>(-Delta Delta C(T))</sup> Method. Methods (San Diego, Calif) 25: 402-408 doi:

10.1006/meth.2001.1262

Lowe TM, Eddy SR (1997) tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res* 25: 955-964 doi:

Moore CM, Gaballa A, Hui M, Ye RW, Helmann JD (2005) Genetic and physiological responses of *Bacillus subtilis* to metal ion stress. *Mol Microbiol* 57: 27-40 doi: 10.1111/j.1365-2958.2005.04642.x

Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature methods* 5: 621-628 doi: 10.1038/nmeth.1226

Nies DH (1992) Resistance to cadmium, cobalt, zinc, and nickel in microbes. *Plasmid* 27: 17-28 doi:

Nies DH, Nies A, Chu L, Silver S (1989) Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. *Proceedings of the National Academy of Sciences of the United States of America* 86: 7351-7355 doi:

Nucifora G, Chu L, Misra TK, Silver S (1989) Cadmium resistance from *Staphylococcus aureus* plasmid pI258 cadA gene results from a cadmium-efflux ATPase. *Proceedings of the National Academy of Sciences of the United States of America* 86: 3544-3548 doi:

Op De Beeck M, Lievens B, Busschaert P, Rineau F, Smits M, Vangronsveld J, Colpaert JV (2015) Impact of metal pollution on fungal diversity and community structures. *Environmental Microbiology* 17: 2035-2047 doi: doi:10.1111/1462-2920.12547

Parsons C, Lee S, Jayeola V, Kathariou S (2017) Novel Cadmium Resistance Determinant in *Listeria monocytogenes*. *Applied and environmental microbiology* 83

Peng W, Li X, Song J, Jiang W, Liu Y, Fan W (2018) Bioremediation of cadmium- and zinc-contaminated soil using *Rhodobacter sphaeroides*. *Chemosphere* 197: 33-41 doi: 10.1016/j.chemosphere.2018.01.017

Reddy MS, Kour M, Aggarwal S, Ahuja S, Marmeisse R, Fraissinet-Tachet L (2016) Metal induction of a *Pisolithus albus* metallothionein and its potential involvement in heavy metal tolerance during mycorrhizal symbiosis. *Environmental Microbiology* 18: 2446-2454 doi: doi:10.1111/1462-2920.13149

Rehman MZU, Rizwan M, Hussain A, Saqib M, Ali S, Sohail MI, Shafiq M, Hafeez F (2018) Alleviation of cadmium (Cd) toxicity and minimizing its uptake in wheat (*Triticum aestivum*) by using organic carbon sources in Cd-spiked soil. *Environmental pollution (Barking, Essex : 1987)* 241: 557-565 doi: 10.1016/j.envpol.2018.06.005

Rensing C, Mitra B, Rosen BP (1997a) The *zntA* gene of *Escherichia coli* encodes a Zn(II)-translocating P-type ATPase. *Proceedings of the National Academy of Sciences of the United States of America* 94: 14326-14331 doi:

Rensing C, Pribyl T, Nies DH (1997b) New functions for the three subunits of the CzcCBA cation-proton antiporter. *J Bacteriol* 179: 6871-6879 doi:

Saha RP, Samanta S, Patra S, Sarkar D, Saha A, Singh MK (2017) Metal homeostasis in bacteria: the role of ArsR-SmtB family of transcriptional repressors in combating varying metal concentrations in the environment. *Biometals* 30: 459-503 doi: 10.1007/s10534-017-0020-3

Satarug S, Baker JR, Urbanjapol S, Haswell-Elkins M, Reilly PEB, Williams DJ, Moore MR (2003) A global perspective on cadmium pollution and toxicity in non-occupationally exposed population. *Toxicology Letters* 137: 65-83 doi: [http://doi.org/10.1016/S0378-4274\(02\)00381-8](http://doi.org/10.1016/S0378-4274(02)00381-8)

Silver S, Phung LT (1996) Bacterial heavy metal resistance: new surprises. *Annual review of microbiology* 50: 753-789 doi: 10.1146/annurev.micro.50.1.753

Solovieva IM, Entian KD (2002) Investigation of the *yvgW* *Bacillus subtilis* chromosomal gene involved in Cd(2+) ion resistance. *FEMS microbiology letters* 208: 105-109 doi: 10.1111/j.1574-6968.2002.tb11068.x

Wang A, Crowley DE (2005) Global gene expression responses to cadmium toxicity in *Escherichia coli*. *J Bacteriol* 187: 3259-3266 doi: 10.1128/jb.187.9.3259-3266.2005

Ye J, Kandededara A, Martin P, Rosen BP (2005) Crystal structure of the *Staphylococcus aureus* pl258 CadC Cd(II)/Pb(II)/Zn(II)-responsive repressor. *J Bacteriol* 187: 4214-4221 doi: 10.1128/jb.187.12.4214-4221.2005

Yoon KP, Misra TK, Silver S (1991) Regulation of the *cadA* cadmium resistance determinant of *Staphylococcus aureus* plasmid pl258. *J Bacteriol* 173: 7643-7649 doi:

Yoon KP, Silver S (1991) A second gene in the *Staphylococcus aureus cadA* cadmium resistance determinant of plasmid pl258. *J Bacteriol* 173: 7636-7642 doi:

Yu X, Wei S, Yang Y, Ding Z, Wang Q, Zhao J, Liu X, Chu X, Tian J, Wu N, Fan Y (2018) Identification of cadmium-binding proteins from rice (*Oryza sativa* L.). *International journal of biological macromolecules* 119: 597-603 doi: 10.1016/j.ijbiomac.2018.07.190

Yu X, Xu J, Liu X, Chu X, Wang P, Tian J, Wu N, Fan Y (2015) Identification of a highly efficient stationary phase promoter in *Bacillus subtilis*. *Scientific reports* 5: 18405 doi: 10.1038/srep18405

## Tables

Due to technical limitations, Tables 1 & 2 are only available as a download in the supplemental file section.

## Figures

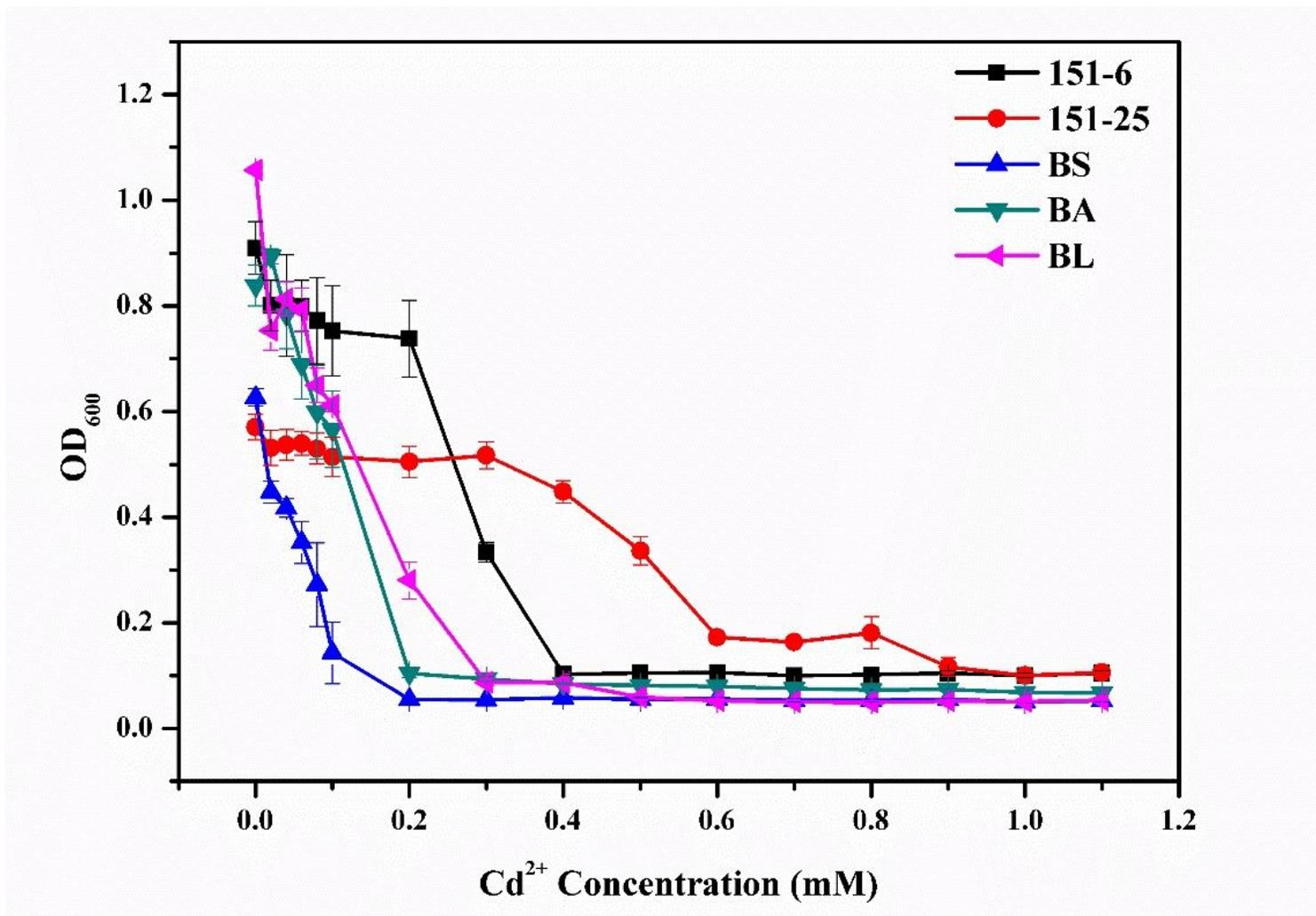
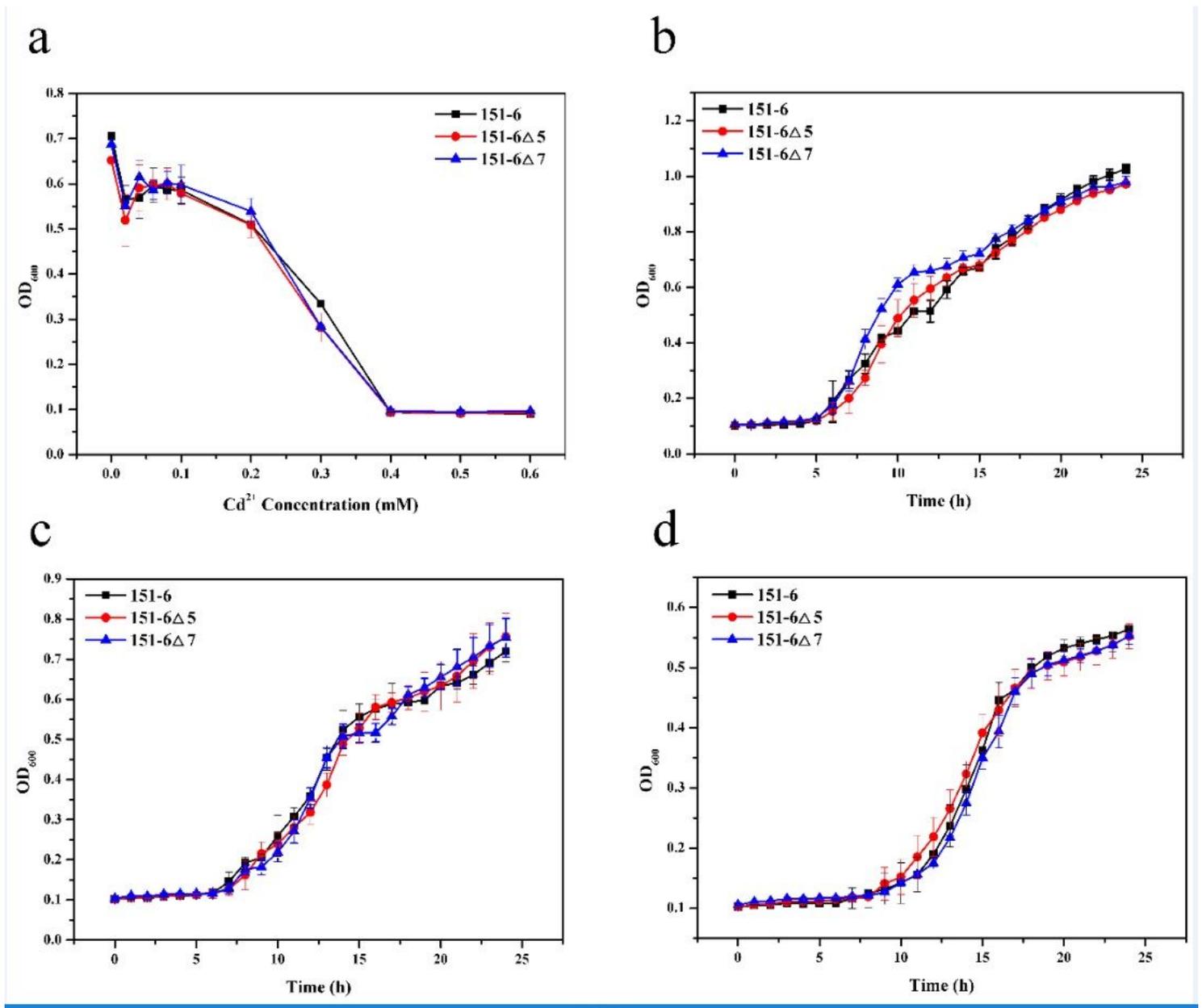


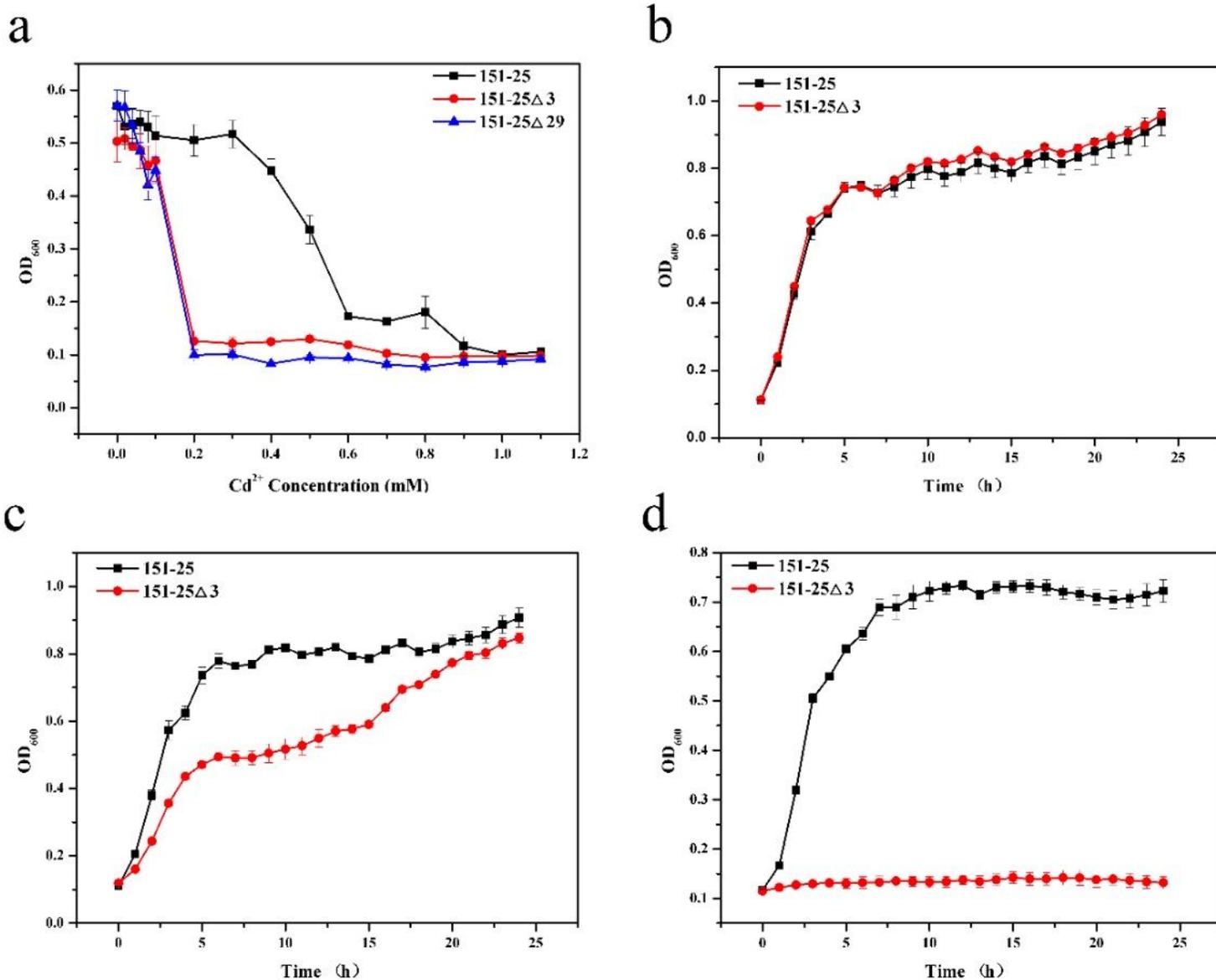
Figure 1

Evaluation of minimum inhibitory concentration of Cd<sup>2+</sup> (MIC-Cd) for *Bacillus* sp. strains (151-6, 151-25, *B. subtilis* WB600 (BS), *B. amyloliquefaciens* (BA) and *B. licheniformis* WX-02 (BL)) at varying concentrations of Cd<sup>2+</sup> (0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 and 1.1 mM).



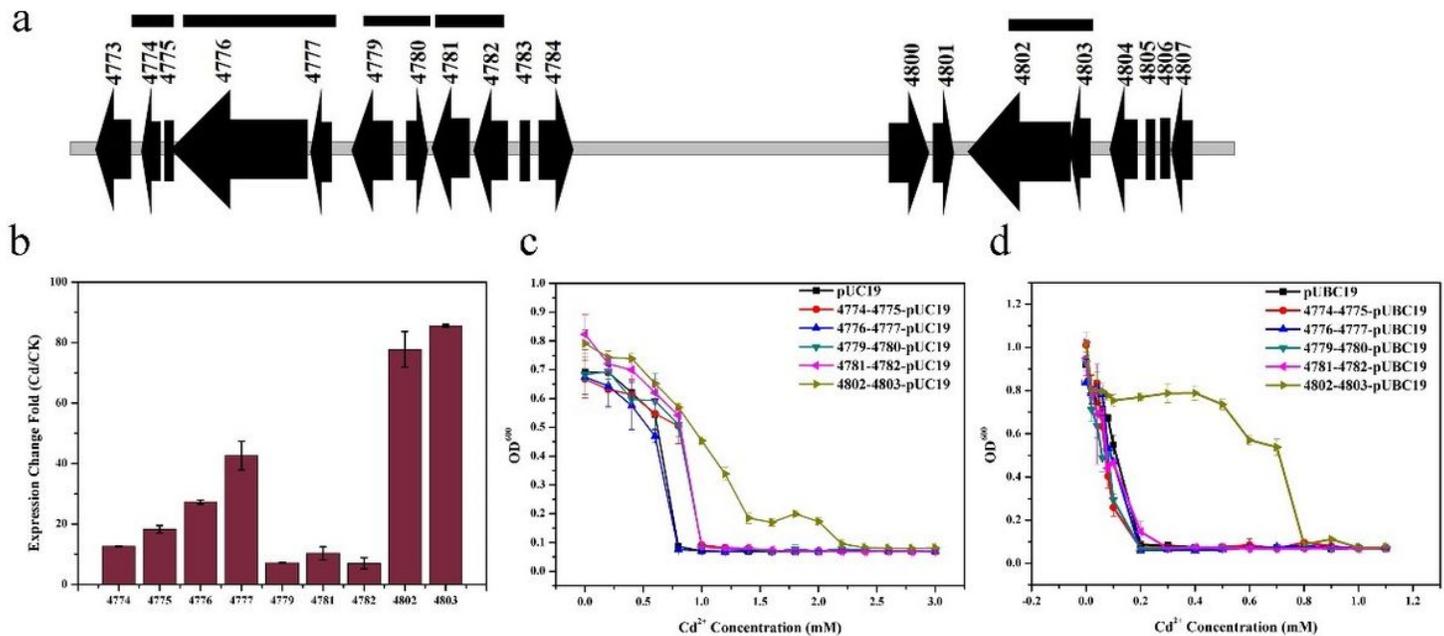
**Figure 2**

MIC-Cd and growth curves for the plasmid p6-eliminated strains 151-6 $\Delta$ 5, 151-6 $\Delta$ 7 and wild strain *B. vietnamsis* 151-6. (a) Evaluation of MIC-Cd for 151-6 $\Delta$ 5, 151-6 $\Delta$ 7 and 151-6 at varying concentrations of Cd<sup>2+</sup> (0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mM).. (b, c and d) Growth curve for 151-6 $\Delta$ 5, 151-6 $\Delta$ 7 and 151-6 at various concentrations of Cd<sup>2+</sup>. (b) 0 mM Cd<sup>2+</sup> added. (c) 0.1 mM Cd<sup>2+</sup> added. (d) 0.2 mM Cd<sup>2+</sup> added.



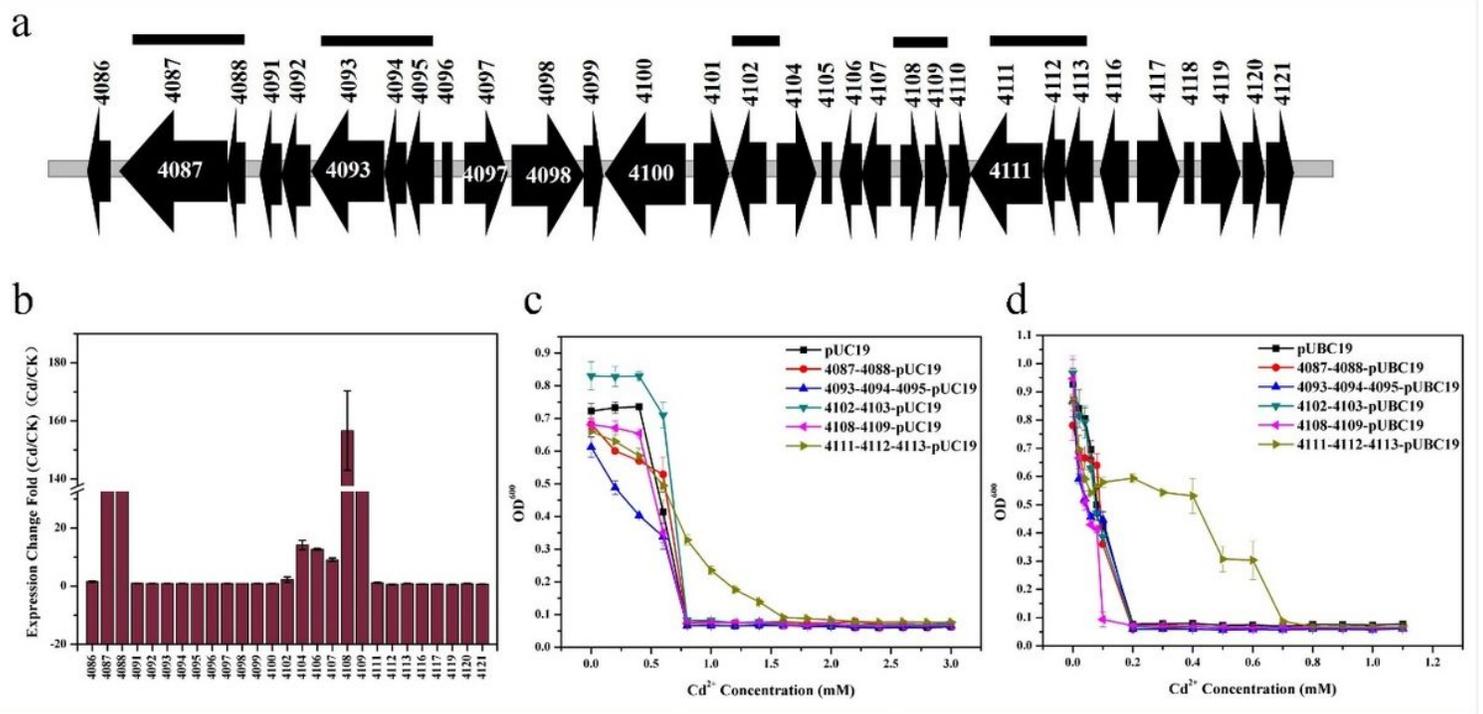
**Figure 3**

MIC-Cd and growth curves for the plasmid p25-eliminated strains 151-25Δ3 and 151-25Δ29, and the wild-type strain *B. marisflavi* 151-25. (a) Evaluation of MIC-Cd for 151-25Δ3, 151-25Δ29 and 151-25 at varying concentrations of Cd<sup>2+</sup> (0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 and 1.1 mM). (b, c and d) Growth curves for 151-25Δ3, 151-25Δ29 and 151-25 at various concentrations of Cd<sup>2+</sup>. (b) 0 mM Cd<sup>2+</sup> added. (c) 0.1 mM Cd<sup>2+</sup> added. (d) 0.5 mM Cd<sup>2+</sup> added.



**Figure 4**

Genetic organization and transcription analysis of up-regulated genes based on RNA sequencing and examination of MIC-Cd for recombinant *E. coli* and *B. subtilis* containing the gene fragments of p25. (a) Schematic representation of loci in the plasmid p25 fragment. Thick gray line, plasmid p25 DNA; filled arrows and squares, 19 open reading frames with corresponding gene sizes; black horizontal line, overexpressed gene fragments. (b) Transcription analysis of the plasmid p25 genes orf4774, 4775, 4776, 4777, 4779, 4781, 4782, 4802 and 4803 in *B. marisflavi* 151-25 cultured with 0.1 mM Cd<sup>2+</sup> -Cd in comparison with a culture grown in the absence of Cd<sup>2+</sup> (CK) using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Transcript levels of tested genes were normalized to the 16S rRNA gene. (c) Determination of MIC-Cd for recombinant *E. coli* containing the vectors pUC19 (negative control), 4774-4775-pUC19, 4776-4777-pUC19, 4779-4780-pUC19, 4781-4782-pUC19 and 4802-4803-pUC19 with varying concentrations of Cd<sup>2+</sup> (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0 mM). (d) Determination of MIC-Cd for recombinant *B. subtilis* containing the vectors pUBC19 (negative control), 4774-4775-pUBC19, 4776-4777-pUBC19, 4779-4780-pUBC19, 4781-4782-pUBC19 and 4802-4803-pUBC19 with varying concentrations of Cd<sup>2+</sup> (0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 and 1.1 mM).



**Figure 5**

Genetic organization and transcription analysis of insert genes in fosmid clone B2 and examination of MIC-Cd for recombinant *E. coli* and *B. subtilis* containing the gene fragments of *B. vietnamsis* 151-6. (a) Schematic representation of the insert fragments loci of fosmid clone B2. Thick gray line, *B. vietnamsis* 151-6 chromosomal DNA; filled arrows and square, 31 open reading frames with corresponding gene size; black horizontal line, overexpressed gene fragments. (b) Transcription analysis of the insert genes in fosmid clone B2 in *B. vietnamsis* 151-6 cultured with 0.1 mM Cd<sup>2+</sup> (Cd) in comparison with a culture grown in the absence of Cd<sup>2+</sup> (CK) using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Transcript levels of tested genes were normalized to the 16S rRNA gene. (c) Determination of MIC-Cd for recombinant *E. coli* containing the vectors pUC19 (negative control), 4087-4088-pUC19, 4093-4094-4095-pUC19, 4102-4103-pUC19, 4108-4109-pUC19 and 4111-4112-4113-pUC19 with varying concentrations of Cd<sup>2+</sup> (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.2, 2.4, 2.6, 2.8 and 3.0 mM). (d) Determination of MIC-Cd for recombinant *B. subtilis* containing the vectors pUBC19 (negative control), 4087-4088-pUBC19, 4093-4094-4095-pUBC19, 4102-4103-pUBC19, 4108-4109-pUBC19 and 4111-4112-4113-pUBC19 with varying concentrations of Cd<sup>2+</sup> (0, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 and 1.1 mM).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [supplement1.pdf](#)
- [supplement2.pdf](#)