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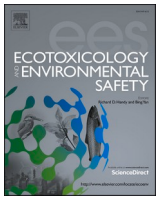

Cadmium-absorptive *Bacillus vietnamensis* 151–6 reduces the grain cadmium accumulation in rice (*Oryza sativa* L.): Potential for cadmium bioremediation

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ABSTRACT

Microbial bioremediation of heavy metal-polluted soil is a promising technique for reducing heavy metal accumulation in crops. In a previous study, we isolated *Bacillus vietnamensis* strain 151–6 with a high cadmium (Cd) accumulation ability and low Cd resistance. However, the key gene responsible for the Cd absorption and bioremediation potential of this strain remains unclear. In this study, genes related to Cd absorption in *B. vietnamensis* 151–6 were overexpressed. A thiol-disulfide oxidoreductase gene (*orf4108*) and a cytochrome C biogenesis protein gene (*orf4109*) were found to play major roles in Cd absorption. In addition, the plant growth-promoting (PGP) traits of the strain were detected, which enabled phosphorus and potassium solubilization and indole-3-acetic acid (IAA) production. *Bacillus vietnamensis* 151–6 was used for the bioremediation of Cd-polluted paddy soil, and its effects on growth and Cd accumulation in rice were explored. The strain increased the panicle number (114.82%) and decreased the Cd content in rice rachises (23.87%) and grains (52.05%) under Cd stress, compared with non-inoculated rice in pot experiments. For field trials, compared with the non-inoculated control, the Cd content of grains inoculated with *B. vietnamensis* 151–6 was effectively decreased in two cultivars (low Cd-accumulating cultivar: 24.77%; high Cd-accumulating cultivar: 48.85%) of late rice. *Bacillus vietnamensis* 151–6 encoded key genes that confer the ability to bind Cd and reduce Cd stress in rice. Thus, *B. vietnamensis* 151–6 exhibits great application potential for Cd bioremediation.

1. Introduction

Soil heavy metal (HM) contamination is a growing problem worldwide. HMs not only destroy the diversity of soil microorganisms and reduce the yield of crops but also endanger human health through the food chain (Lin et al., 2016; McLaughlin et al., 1999). Cadmium (Cd) is one of the most toxic HMs even at lower concentrations of 0.001–0.1 mg/L (Singh et al., 2016) and has been classified as a human carcinogen (Cui et al., 2021; Zhu and Costa, 2020). Rice is an important source of carbohydrates for most Asians, especially in China. However, it has been reported that, on average, 12 million tons of rice in China are contaminated with Cd annually (Sun et al., 2016; Zhao et al., 2021). According to the Codex Alimentarius Commission of the Food and Agriculture Organization/World Health Organization, the maximum permissible concentration (MPC) of Cd in polished rice is 0.4–0.5 mg/kg (CODEX STAN 193–1995, revised 2010). In China, the safe Cd limit in rice is 0.2 mg/kg (GB2762–2012) (Liu et al., 2017; Wang et al., 2019, 2016). Therefore, reducing the accumulation of Cd in rice grown in polluted cropland soil is essential for food safety, and developing treatment strategies for Cd-contaminated paddy soils is urgent.

Over the years, many chemical and physical methods have been used for Cd pollution remediation. However, the drawbacks of these methods, such as generating chemical waste, complicated downstream treatment processes, and high costs, make them inefficient (Alotaibi et al., 2021;

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Liu et al., 2018). Microbial remediation, especially using bacteria, has drawn increasing attention due to the efficiency and feasibility of applying microbes to decontaminate soil (Alotaibi et al., 2021). Bacteria can detoxify metals via general resistance mechanisms, valence transformation, biosorption, extracellular chemical precipitation, and efflux mechanisms (Liu et al., 2018). Recently, many efforts have been dedicated to the isolation of Cd-resistant and plant growth-promoting bacteria (PGPB) to reduce the migration and absorption of metals by plants. For example, Thanwisai et al. (2022) reported that the application of Cd-tolerant *Cupriavidus taiwanensis* KKU2500–3 reduced Cd accumulation in the grains of KDML105 rice and promoted rice growth. Shan et al. (2020) found that the inoculation of Cd-resistant *Citrobacter* sp. XT1–2–2 in soil not only decreased Cd uptake by rice but also significantly increased rice biomass. Wang et al. (2020) showed that the addition of *Burkholderia* sp. Y4 reduced Cd accumulation in the roots, shoots, and grains of two rice cultivars. These previous studies have shown that various Cd-resistant bacterial strains can be used to remediate Cd-contaminated paddy soils by interacting with HMs.

Bacillus spp. strains are well known for their Cd resistance, plant growth-promoting (PGP) properties, and comprehensive application for the bioremediation of Cd contamination in a variety of environments (Goyal et al., 2019; Miljakovic et al., 2020). Various reported mechanisms through which *Bacillus* spp. act as PGPB include phosphorus solubilization and siderophore, phytohormone, and hydrolase production (Blake et al., 2021; Setiawati et al., 2022). For example, *B. koreensis* 181–22, a PGP endophytic bacterium, has been used as a potential biofertilizer for the bioremediation of Cd-contaminated paddy soils (Zhou et al., 2021). The Cd-resistant rhizobacterium *B. cereus* M4 produces the phytohormone IAA, promotes growth, and reduces Cd accumulation in rice (Wang et al., 2019). As effective PGPB, *B. subtilis* and *B. cereus* also promote rice growth and reduce Cd absorption and toxicity in rice (Treesubsuntorn et al., 2018). In previous studies, *B. vietnamensis* 151–6 and *B. marisflavi* 151–25, isolated from Cd-polluted arable soil, exhibited significant differences in Cd resistance and Cd absorption (Yu et al., 2021). The Cd resistance mechanism of the two strains was explored in another previous work (Yu et al., 2020). In addition, *B. vietnamensis* 151–6 showed a higher Cd removal rate (91.22%) under the same culture conditions than strain 151–25 (22.80%) (Yu et al., 2021). The Cd accumulation ability of the two strains was explored in this work, and the results showed that strain 151–6 had a significantly higher Cd accumulation ability than strain 151–25. Thus, *B. vietnamensis* 151–6 has more application potential in Cd remediation than 151–25. However, the Cd removal/remediation mechanism, PGP traits, and the application of the strain 151–6 to soil remediation, particularly in Cd-polluted paddy soil, have not been investigated.

In this paper, the potential bioremediation mechanisms of *B. vietnamensis* 151–6 were explored, and the effects of strain 151–6 on rice growth and Cd accumulation in Cd-polluted soil were investigated in pot experiments and field trials. Specifically, this study aimed to investigate 1) the potential functional genes related to Cd absorption in *B. vietnamensis* 151–6; 2) the physiological and biochemical indices and PGP traits of *B. vietnamensis* 151–6; and 3) the effect on rice growth and Cd uptake in rice in the presence of *B. vietnamensis* 151–6.

2. Materials and methods

2.1. Cellular distribution of Cd in B. vietnamensis 151–6 and B. marisflavi 151–25

Previous work reported that the Cd removal rates of *B. vietnamensis* 151–6 and *B. marisflavi* 151–25 grown in Luria–Bertani (LB) liquid medium supplemented with 0.1 mM $CdCl_2$ and shaken at 200 rpm at 30 °C for 24 h were 91.22% and 22.80%, respectively (Yu et al., 2021). To further determine the cellular distribution of Cd accumulated in the two strains, the Cd concentrations between the suspension, cell wall, and cytoplasm of the test strains were determined using the method described by McEldowney (2000). Briefly, the cells were grown in LB liquid medium supplemented with 0.1 mM $CdCl_2$ and shaken at 200 rpm at 30 °C for 72 h. Cell suspensions were sampled every 12 h, centrifuged at 12,000 rpm for 10 min, and analyzed to determine the Cd concentration. First, culture supernatants were collected to determine the extracellular Cd concentration. The bacterial precipitate was then washed with 2 mL 0.005 M ethylenediaminetetraacetic acid (EDTA) (3 ×). After centrifugation, the EDTA extract and the washed bacteria pellet were reacted with concentrated HNO_3 under reflux at 180 °C for 30 min to enable the cell wall and cytoplasmic Cd levels to be determined. The Cd concentration of each sample was measured using atomic absorption spectrophotometry (Z-2000, Hitachi, Japan).

2.2. Construction of gene heterologous expressional plasmids and determination of Cd removal efficiency and Cd resistance in recombinant strains

In the previous study, RNA sequencing analysis and qRT-PCR experiments were performed for *B. vietnamensis* 151–6. Moreover, to identify the key Cd resistance genes for strain 151–6, five gene clusters (*orf4807–4088*, *orf-4093–4094–4095*, *orf4102–4103*, *orf4108–4109*, and *orf4111–4112–4113*; GenBank Accession No. CP047394.1) were constructed in plasmid pUC19 and overexpressed in *Escherichia coli* (*E. coli*) (Yu et al., 2020).

In this study, to further verify whether the Cd-binding ability of strain 151–6 was conferred by these genes, the Cd removal rates of the above five recombinant strains were evaluated according to the method of Yu et al. (2021). In addition, recombinant pUC19 plasmids harboring *orf4108* and *orf4109* were constructed. The *orf4108* and *orf4109* genes, amplified from the *B. vietnamensis* 151–6 genome with the corresponding primers (Table 1), were ligated into pUC19 with the same cohesive end. The ligation mixture was used to transform *E. coli* TOP10, and the correct recombinant plasmids were identified through colony PCR with M13-F/M13-R primers (Table 1). Then, the Cd removal rates of the two recombinant strains were evaluated using atomic absorption spectrophotometry (Yu et al., 2021).

To evaluate the Cd tolerance of the recombinant strains, the minimum inhibitory concentration (MIC) of Cd was measured using a method described in a previous study (Yu et al., 2020).

2.3. Characterization of B. vietnamensis 151–6

The physiological and biochemical indices and PGP behaviors of *B. vietnamensis* 151–6 were assessed. The PGP behaviors focused on soluble phosphate, soluble potassium, and phytohormone production (IAA).

2.3.1. Physiological and biochemical indices

The physiological and biochemical indices of *B. vietnamensis* 151–6 were measured according to the instructions provided by BIOLOG GEN III.

2.3.2. Mineral phosphate solubilization activity

Mineral phosphate solubilization activity was evaluated according to the method of Kuklinsky-Sobral et al. (2004). The test strain was

Table 1
Primers used in this study.

Primer	Sequence (5′−3′)[a]
4108-F	ata*GAGCT*CAAAAGTACACCTCTTCAAAATTATT
4108-R	ata*TCTAGA*TTATTGTTCTGGCTTGATCA
4109-F	ata*GAGCTC*ATCCCGACGACATTCCTTAT
4109-R	ata*TCTAGAC*TAGAATCCCGTAAAACCAC
M13-F	CGCCAGGGTTTTCCCAGTCACGAC
M13-R	AGCGGATAACAATTTCACACAGGA

[a] Restriction sites are indicated by italic characters.

cultured overnight in a 3 mL LB liquid medium. Then, 1% microbial solution was added to 100 mL Pikovskaya's medium and shaken at 200 rpm at 37 °C for 7 days. The soluble phosphate content was determined using the molybdenum-blue method (Murphy and Riley, 1962).

2.3.3. Determination of soluble potassium content

Quantitative testing of soluble potassium used 50 mL of Alexandrov media (Keshavarz Zarjani et al., 2013). A single colony of *B. vietnamensis* 151–6 was cultured overnight in 3 mL LB broth. Then, a 1% test culture was used to inoculate Alexandrov media and incubated at 37 °C for 72 h. The soluble K content was detected with a Z-2000 Atomic Absorption Spectrophotometer (Hitachi, Japan) (Parmar and Sindhu, 2013).

2.3.4. IAA production

IAA production was determined using a modified qualitative method described by Gordon and Weber (1951). The test strain was cultured overnight in a 3 mL LB liquid medium. Then, a 1% test culture was used to inoculate LB broth supplemented with 0.5 mg/mL L-tryptophan and incubated at 37 °C for 72 h. The IAA concentrations in the culture suspensions were determined using a SpectraMax M2 spectrophotometer (Molecular Devices, USA).

2.4. Pot experiments

Pot experiments were designed to determine the effect of isolated indigenous *B. vietnamensis* 151–6 on rice growth. Soil samples were obtained from a Cd-polluted paddy field (0.93 mg/kg) in XiangTan City, Hunan Province, China. The samples were naturally dried, and impurities were eliminated. The soils were pulverized before use. Rice seedlings (*Oryza sativa* L. T705) were surface-sterilized in 10% sodium hypochlorite for 10 min, washed several times with sterile distilled water, soaked, and germinated for 2 days in a 30 °C incubator. The seedlings were then grown for 28 days in Cd-free MS medium (Solarbio, Beijing). Before they were transplanted into pots containing Cd-polluted soil, the rice seedling roots were inoculated with a bacterial suspension for 0.5 h.

Bacillus vietnamensis 151–6 was used for the pot experiments. The bacterium was cultured in liquid LB medium at 37 °C for 48 h. Cells were collected via centrifugation at 8000 rpm for 10 min and resuspended in sterile distilled water (ca. 1×10^9 cfu/mL). The rice roots were dipped in cell suspensions (10 mL/pot) for 0.5 h and then transplanted into the pots, after which the remaining bacterial solution was added to the soil. The following three growing conditions were devised: 1) Adding bacteria and Cd: The bacterial culture broth and 1.5 mg/kg Cd were added to the Cd-contaminated soil at the transplanting, tillering, and grain-filling stages of *O. sativa* L. T705. The total supplemental amount of Cd was 4.5 mg/kg. 2) Addition of Cd and no bacteria: Soil with no inoculation bacteria but with the addition of 4.5 mg/kg Cd at the three growing stages of rice plants was used as the control. 3) No added bacteria or Cd: Normal soil with no added bacteria or Cd was used as the negative control. Each experiment was performed in triplicate. For each treatment, 9 kg of air-dried raw soil was transferred to each pot, which was 30.5 cm in diameter and 30 cm in height.

2.5. Field trials

Rice is planted twice a year in Hunan Province, China. Early rice is generally planted in February or March and harvested in June or July, depending on the local temperature. Late rice is planted from March to June and harvested in October or November. Field trials (N: 27°52′, E: 111°50′, Cd: 0.48 mg/kg) were conducted in 2018 during the early and late rice-growing seasons. Seeds of two *O. sativa* L. cultivars, 'Xiangwanxian 12′ (low Cd-accumulating cultivar) and 'Huazhan' (high Cd-accumulating cultivar), were used in the study. During the field experiment, the bacterial treatment was designed to investigate the effects of bacteria on the repression of Cd absorption. The treatment was conducted in a completely randomized block design with three replications. Each plot was 30.0 m^2 with a length of 5.0 m and a width of 6.0 m. Two treatments were conducted as follows: 1) Adding bacteria: *B. vietnamensis* strain 151–6 was cultured and pretreated as described in Sections 2.4, and 2 L bacterial suspensions were applied at the three stages (the transplanting, tillering, and filling stages) of *O. sativa* L. Xiangwanxian 12 and *O. sativa* L. Huazhan in each plot; 2) no bacteria. The management of each field was the same as that used in local production. Each field experiment was performed in triplicate.

2.6. Measurement of Cd concentrations

The rice plants were harvested at maturity. The harvested roots, rachises, and seeds were dried in a 65 °C incubator for 3 days. Samples weighing approximately 0.50 g were digested with HNO_3 and 3 mL HF (Yu et al., 2021), and the Cd content in the digests was detected using an inductively coupled plasma mass spectrometry instrument (Agilent Technologies, Japan). The effects of indigenous *B. vietnamensis* 151–6 on Cd accumulation in rice in the pot and field experiments were analyzed.

2.7. Statistical analyses

Data were summarized with the mean of three replicates and standard deviation (SD) of the mean. Data were analyzed via one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test.

3. Results

3.1. Cellular distribution of Cd content in B. vietnamensis 151–6 and B. marisflavi 151–25

The cellular distributions of Cd accumulation in *B. vietnamensis* 151–6 and *B. marisflavi* 151–25 are summarized in Table 2. In Table 2, the residual Cd content in the media supernatant of strain 151–6 gradually decreased with the extension of the incubation time, while the accumulated Cd content in the cytoplasm gradually increased. At 72 h, approximately 44.38% of the Cd content was present as a suspension, 54.49% was present in the cytoplasm, and only 1.13% was bound to the cell wall. In contrast, strain 151–25 had a much weaker Cd accumulation capacity, in which 90.11% of the Cd ions were distributed extracellularly. At 36 h, the percentage of the intracellular accumulation of Cd ions for strain 151–25 was maximal at only 14.99%, and the ratio of the residual Cd content in the cytoplasm at this time was 84.56% (Table 2). Strain 151–6 exhibited a higher Cd content of cellular distribution than strain 151–25, demonstrating that 151–6 possesses a higher Cd accumulation ability. Therefore, strain 151–6 was selected to investigate the Cd accumulation mechanism and Cd-binding-related genes.

3.2. A gene fragment including orf4108 and orf4109 confers high Cd removal in B. vietnamensis 151–6

To determine the genes related to the Cd-binding ability of *B. vietnamensis* 151–6, the Cd removal rates of five recombinant *E. coli* strains (containing *orf4087–4088*, *orf-4093–4094–4095*, *orf4102–4103*, *orf4108–4109*, and *orf4111–4112–4113*) were evaluated. As shown in Fig. 1, the gene clusters of *orf4087–4088*, *orf-4093–4094–4095*, *orf4102–4103*, *orf4108–4109*, and *orf4111–4112–4113* enabled recombinant *E. coli* to exhibit a higher Cd removal rate than the negative control strain (containing plasmid pUC19) in LB media with the addition of 0.05 and 0.1 mM $CdCl_2$ for 24 h. In particular, the Cd removal rates of *E. coli* cells containing the recombinant plasmid 4108–4109-pUB19 were 44.21% and 21.59% in 0.05 and 0.1 mM $CdCl_2$ LB media, respectively, which were 5.71- and 3.32-fold higher than the rates of the negative cells, respectively (Fig. 1 and Table S1). This result indicates that the gene cluster consisting of *orf4108* (thiol-disulfide oxidoreductase gene)

Table 2
Cellular distribution of Cd content in *B. vietnamensis* 151–6 and *B. marisflavi* 151–25.

Strains		12 h	24 h	36 h	48 h	60 h	72 h
151–6	Suspension (%)	98.06 ± 0.20	93.56 ± 0.37	87.01 ± 2.42	66.47 ± 0.54	48.99 ± 2.21	44.38 ± 0.89
	Cell Wall (%)	0.17 ± 0.01	0.22 ± 0.01	0.31 ± 0.00	0.64 ± 0.04	0.82 ± 0.08	1.13 ± 0.07
	Cytoplasm (%)	1.77 ± 0.19	6.22 ± 0.38	12.68 ± 1.43	32.89 ± 0.14	50.19 ± 3.02	54.49 ± 2.13
151–25	Suspension (%)	93.30 ± 0.35	87.29 ± 0.82	84.56 ± 0.51	86.70 ± 0.79	89.96 ± 0.51	90.11 ± 0.19
	Cell Wall (%)	0.56 ± 0.02	0.42 ± 0.06	0.45 ± 0.04	0.78 ± 0.01	1.00 ± 0.07	1.08 ± 0.09
	Cytoplasm (%)	6.14 ± 0.37	12.29 ± 0.88	14.99 ± 0.55	12.52 ± 0.63	9.04 ± 0.20	8.81 ± 0.11

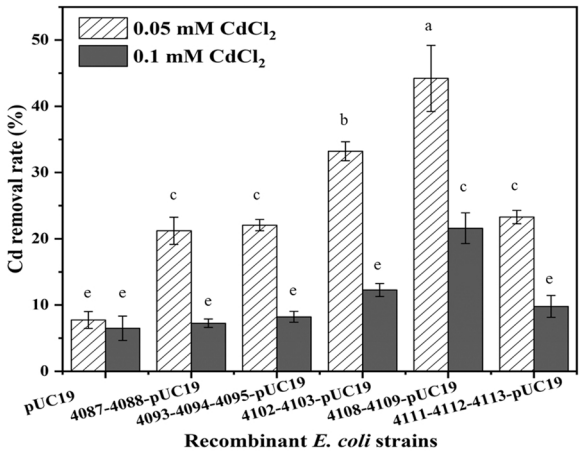

Fig. 1. Determination of the Cd removal rate of recombinant *E. coli* strains in LB medium supplemented with 0.05 and 0.1 mM $CdCl_2$ for 24 h, respectively. The bar represents ± SD (n = 3). Data in columns indexed with the same letters do not differ significantly according to Fisher's LSD test ($p < 0.05$).

and *orf4109* (cytochrome C biogenesis protein CcdA gene) conferred the major Cd absorption ability of *B. vietnamensis* 151–6.

To further identify the function of *orf4108* and *orf4109* in the Cd absorption ability of *B. vietnamensis* 151–6, two recombinant *E. coli* strains that contained plasmids orf4108-pUC18 and orf4109-pUC19 were constructed. The Cd removal rates of the four recombinant strains (including pUC19-*E. coli* and orf4108–4109-pUC19-*E. coli*) were continuously evaluated. As shown in Fig. 2 A and Table S2, the gene cluster of *orf4108–4109* allowed recombinant *E. coli* to remove more Cd than *orf4109*, *orf4108*, and the negative control in the presence of 0.05 and 0.1 mM $CdCl_2$, in the order of orf4108–4109-pUC19 > orf4109-pUC19 > orf4108-pUC19 > pUC19. This finding indicates that both *orf4109* and *orf4108* were related to the Cd absorption ability of *B. vietnamensis* 151–6 and that *orf4109* was more correlated with the Cd absorption ability of 151–6 than *orf4108*. In addition, to determine whether *orf4108*, *orf4109*, and the gene cluster *orf4108–4109* were involved with Cd resistance in 151–6, the Cd-MICs for the above four recombinant strains were evaluated. As shown in Fig. 2B, in liquid media, the strains containing orf4108-pUC19, orf4109-pUC19, and orf4108–4109-pUC19 recombinant plasmids exhibited Cd-MICs similar to those of the negative strain. This result indicates that neither *orf4108* nor *orf4109* was associated with Cd resistance in 151–6. Altogether, these results suggest that the gene fragment of *orf4108–4109* played a major role in the Cd removal ability of *B. vietnamensis* 151–6 but not in the Cd resistance of the strain.

3.3. Detection of the PGP traits and physiological and biochemical indices of *B. vietnamensis* 151–6

To determine whether strain 151–6 had PGP traits, phosphate solubilization, soluble potassium, and phytohormone IAA were detected. As shown in Table 3, *B. vietnamensis* 151–6 could dissolve phosphate and potassium and produce IAA. Specifically, *B. vietnamensis* 151–6 produced 35.61 ± 3.97 mg/L solubilized mineral phosphate after 7 days of culture in Pikovskaya's medium, and this strain produced 5.32 ± 0.77 mg/L soluble potassium and 9.05 ± 0.17 mg/L IAA under the corresponding culture conditions for 3 days. These results indicate that *B. vietnamensis* 151–6 has potential PGPB properties.

In addition, BIOLOG GEN III identification technology was used to detect the biochemical reactions and other indices of *B. vietnamensis*

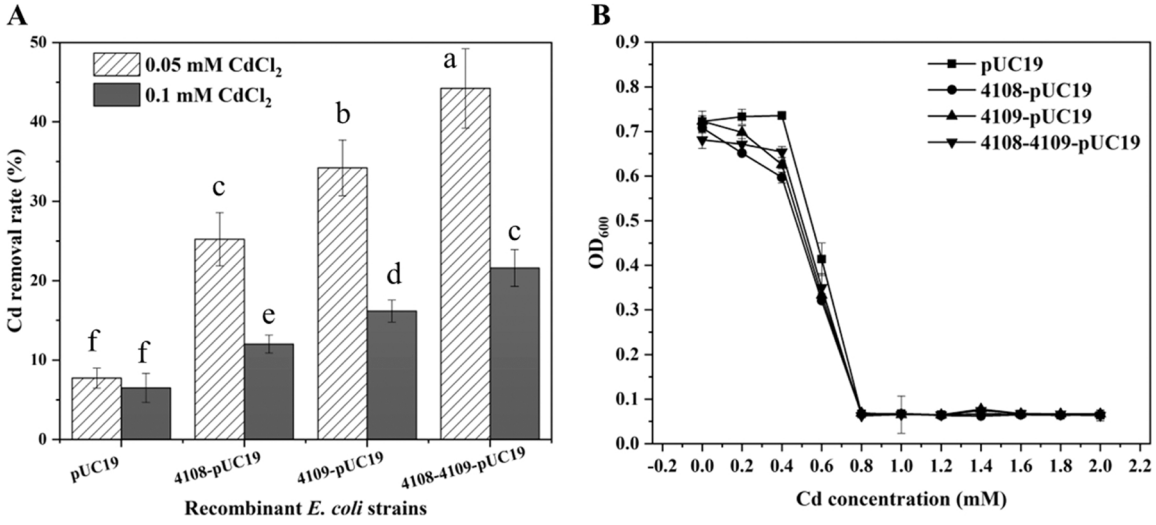

Fig. 2. Determination of the Cd removal rate and Cd-MIC of recombinant *E. coli* strains. (A) Evaluation of the Cd removal rate of recombinant *E. coli* strains in LB medium supplemented with 0.05 and 0.1 mM $CdCl_2$ for 24 h, respectively. (B) Determination of Cd-MIC of recombinant *E. coli* strains at varying concentrations of $CdCl_2$ (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 mM). The bar represents ± SD (n = 3). Data in columns indexed with the same letters do not differ significantly according to Fisher's LSD test ($p < 0.05$).

Table 3
PGP traits and physiological and biochemical indices of *B. vietnamensis* 151–6.

Test	Result	Test	Result	Test	Result
Cell morphology	rhabditiform	myo-Inosito	-	γ-Amino-Butryric Acid	-
solubilize mineral phosphate (mg/L)	35.61 ± 3.97	Glycerol	+	α-Hydroxy-Butyric Acid	-
soluble potassium (mg/L)	5.32 ± 0.77	D-Glucose-6-PO_4	+	β-Hydroxy-D,L ButyricAcid	+
IAA production (mg/L)	9.05 ± 0.17	D-Fructose-6-PO_4	+	α-Keto-Butyric Acid	-
Negative control	-	D-Aspartic Acid	+	Acetoacetic Acid	+
Dextrin	+	D-Serine	-	Propionic Acid	-
D-Maltose	\	Gelatin	+	Acetic Acid	+
D-Trehalose	\	Glycyl-L-Proline	\	Formic Acid	-
D-Cellobiose	-	L-Alanine	-	Positive Control	+
Gentiobiose	-	L-Arginine	+	pH 6	-
Sucrose	-	L-Aspartic Acid	+	pH 5	-
D-Turanose	-	L-Glutamic Acid	+	1% NaCl	+
Stachyose	-	L-Histidine	+	4% NaCl	+
D-Raffinose	-	L-Pyroglutamic Acid	+	8% NaCl	+
α-D-Lactose	-	L-Serine	+	1% Sodium Lactate	+
D-Melibiose	-	Pectin	+	Fusidic Acid	-
β-Methyl-D-Glucoside	-	D-Galacturonic Acid	-	D-Serine	-
D-Salicin	-	L-Galactonic Acid Lactone	-	Troleandomycin	-
N-Acetyl-D-Glucosamine	+	D-Gluconic Acid	+	Rifamycin SV	-
N-Acetyl-β-D Mannosamine	-	D-Glucuronic Acid	-	Minocycline	-
N-Acetyl-D-Galactosamine	-	Glucuronamid e	-	Lincomycin	-
N-Acetyl Neuraminic Acid	-	Mucic Acid	\	Guanidine HCl	-
α-D-Glucose	+	Quinic Acid	-	Niaproof 4	-
D-Mannose	-	D-Saccharic Acid	-	Vancomycin	-
D-Fructose	+	p-Hydroxy-Phenylacetic Acid	-	Tetrazolium Violet	-
D-Galactose	-	Methyl Pyruvate	\	Tetrazolium Blue	-
3-Methyl Glucose	-	D-Lactic Acid Methyl Ester	-	Nalidixic Acid	-
D-Fucose	-	L-Lactic Acid	+	Lithium Chloride	+
L-Fucose	-	Citric Acid	-	Potassium Tellurite	-
L-Rhamnose	-	α-Keto-Glutaric Acid	-	Aztreonam	+
Inosine	+	D-Malic Acid	-	Sodium Butyrate	+
D-Sorbitol	-	L-Malic Acid	+	Sodium Bromate	-
D-Mannitol	+	Bromo-Succinic Acid	-		
D-Arabitol	-	Tween 40	+		

Note: "+ " indicates positive; "-" indicates negative; "\" indicates boundary.

151–6 with 71 different carbon sources and 23 chemically sensitive substances (Table 3) to verify the characteristics of the strain. *Bacillus vietnamensis* 151–6 used dextrin, N-acetyl-D-glucosamine, α-D-glucose, D-fructose, inosine, D-mannitol, glycerol, D-glucose-6-PO_4, D-fructose-6-PO_4, D-aspartic acid, gelatin, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, pectin, D-gluconic acid, L-lactic acid, L-malic acid, Tween 40, β-hydroxy-D, L butyric acid, acetoacetic acid, and acetic acid. Both the pH 6 and pH 5 tests of *B. vietnamensis* 151–6 were negative, indicating that a low pH environment was not conducive to the growth of the strain. Nevertheless, the 1% NaCl, 4% NaCl, and 8% NaCl tests of *B. vietnamensis* 151–6 were positive, suggesting a certain tolerance to salt.

3.4. Effects of B. vietnamensis 151–6 on the growth of O. sativa L. T705 in pot experiments

Pot experiments were conducted to clarify the effects of *B. vietnamensis* 151–6 during the rice growth period in Cd-polluted soil. Cd treatment significantly inhibited growth in *O. sativa* L. T705 (Fig. 3 A). Adding 4.5 mg/kg Cd remarkably decreased the number of panicles (from 20.67 ± 4.93–9.00 ± 1.95) (Fig. 3 C and Table S4). After inoculation with *B. vietnamensis* 151–6, the growth inhibition of rice was alleviated (Fig. 3 A). The number of panicles (19.33 ± 3.05) recovered (Fig. 3 C and Table S4). There were no remarkable differences in the plant heights of rice among the three growing conditions (T705: 82.66 ± 2.52 cm; T705 + Cd: 76.44 ± 4.07 cm; and T705 + Cd+151–6: 77.89 ± 3.34 cm) (Fig. 3B and Table S3). Thus, inoculation with *B. vietnamensis* 151–6 alleviated the inhibitory effect of Cd on rice growth.

The Cd content in the roots, rachises, and grains of rice is shown in Fig. 3D. Overall, Cd accumulation in the roots was significantly higher than that in the rachises and grain tissues in the three experiments. The Cd content in the three rice tissues in the treatment without Cd addition (T705) was significantly lower than that of the Cd (T705 + Cd) and Cd+ 151–6-treatments (T705 + Cd+151–6) (Table S5). The Cd accumulation was lower in rice roots inoculated with *B. vietnamensis* 151–6 (T705 + Cd+151–6) (479.35 ± 16.74 mg/kg) than in the control (T705 + Cd) (514.69 ± 23.74 mg/kg), exhibiting a difference of 6.87%. Cd content was also less in *B. vietnamensis* 151–6-treated rice than in non-treated rice in the rachises (55.48 ± 4.00 and 72.88 ± 2.42 mg/kg, respectively) and grains (2.28 ± 0.18 and 1.09 ± 0.05 mg/kg, respectively), showing differences of 23.87% and 52.05%, respectively (Table S5). The application of *B. vietnamensis* 151–6 in potted rice soil significantly reduced the Cd content in the rachises and grains of *O. sativa* L. T705.

3.5. Effects of B. vietnamensis 151–6 on the accumulation of rice grains in field trials

Field trials were conducted to further clarify the effects of *B. vietnamensis* 151–6 on Cd uptake in rice grains. The Cd content of the two late rice grain cultivars was higher than that of the two early grain cultivars (Fig. 4). The Cd content in the grains of the two cultivars of early rice was less than the MPC (0.2 mg/kg), except for the grains of the high Cd-accumulating cultivar Huazhan, without inoculation with *B. vietnamensis* 151–6 (0.20 ± 0.018 mg/kg) (Fig. 4 A). However, the Cd content in the grains of the two late cultivars was more than 0.2 mg/kg (Fig. 4B).

Compared to the non-inoculated control, the Cd content of grains inoculated with *B. vietnamensis* 151–6 was decreased in both cultivars of

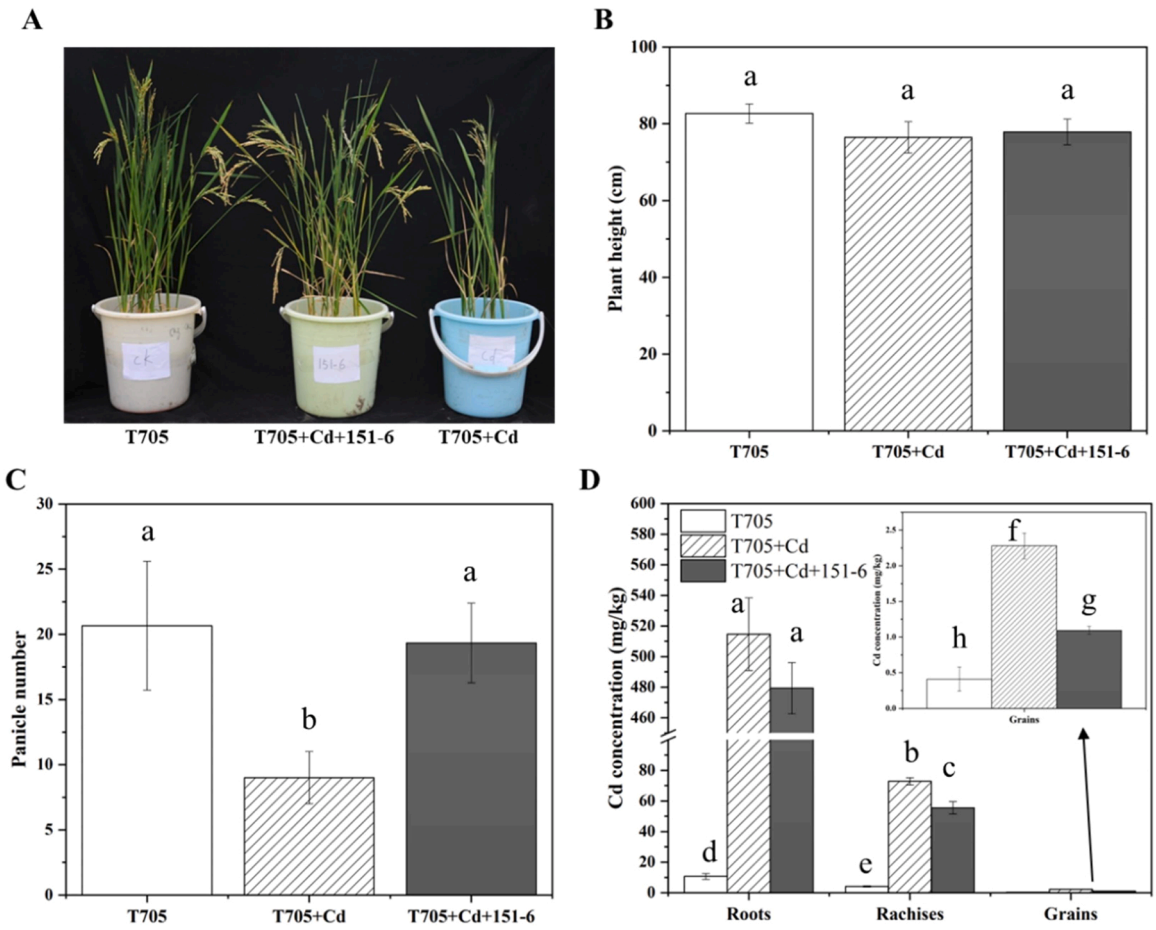

Fig. 3. Effects of *B. vietnamensis* 151–6 on rice (*O. sativa L.* T705) grown in Cd-contaminated soil. (A) Pot experiments conducted throughout the growth period of rice; (B) plant height of rice; (C) number of panicles; and (D) Cd concentrations in different tissues of rice. T705 (no added bacteria or Cd, total Cd 0.93 mg/kg), T705 + Cd (added Cd with no added bacteria, total Cd 5.43 mg/kg), and T705 + Cd+ 151–6 (added bacteria and Cd, total Cd 5.43 mg/kg). The bar represents ± SD (n = 3). Data in columns indexed with the same letters do not differ significantly according to Fisher's LSD test ($p < 0.05$).

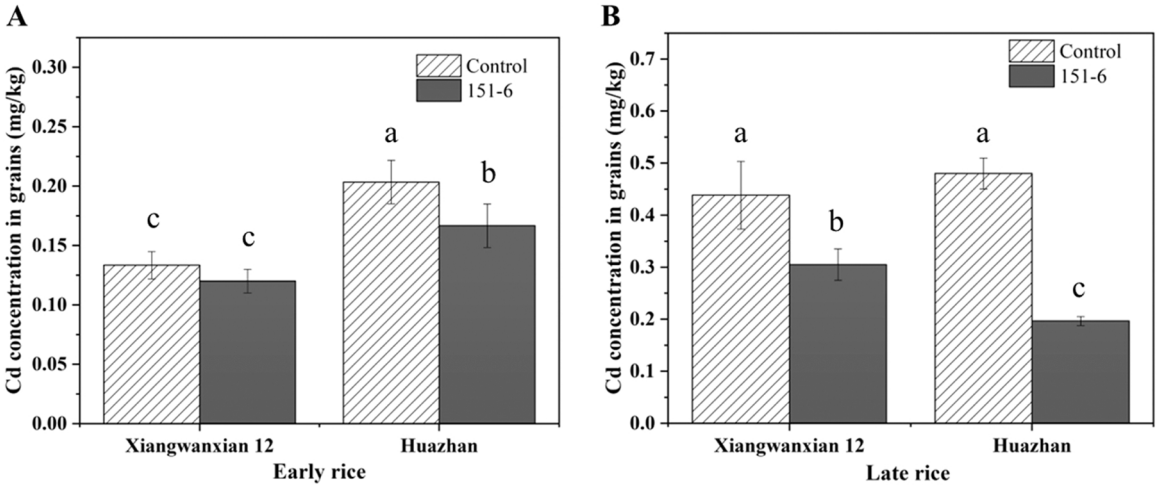

Fig. 4. Cd content in rice grains of *O. sativa* L. 'Xiangwanxian 12′ (low Cd-accumulating cultivar) and 'Huazhan' (high Cd-accumulating cultivar) with or without inoculation of *B. vietnamensis* 151–6. (A) Cd content in the grains of two early rice cultivars; (B) Cd content in the grains of two late rice cultivars. Control (no added bacteria, Cd 0.48 mg/kg); 151–6 (added *B. vietnamensis* 151–6, Cd 0.48 mg/kg). The bar represents ± SD (n = 3). Data in columns indexed with the same letters do not differ significantly according to a t-test ($p < 0.05$).

late rice (Fig. 4B), and the strain reduced the grain Cd content of the early rice cultivar Huazhan (Fig. 4 A). However, there was no significant decrease in the Cd content in early rice cultivar Xiangwanxian 12 (Fig. 4 A and Table S6). In detail, for early rice, inoculation with *B. vietnamensis* 151–6 reduced Cd content in the grains of the low Cd-accumulating cultivar Xiangwanxian 12 and the high Cd-accumulating

cultivar Huazhan by 10.00% and 18.03%, respectively, but the difference of Huazhan was not remarkable ($p > 0.05$) (Fig. 4 A and Table S6). However, for late rice, after inoculation with *B. vietnamensis* 151–6, the Cd content in Xiangwanxian 12 and Huazhan was reduced significantly ($p < 0.05$) by 24.77% and 48.85%, respectively (Fig. 4B and Table S7). These results show that inoculation with *B. vietnamensis* 151–6 was more effective in reducing Cd accumulation in the grains of late rice than in the grains of early rice, and the effect of decreasing grain Cd accumulation in the high Cd-accumulating rice cultivar exceeded that of the low Cd-accumulating rice cultivar. These results indicate that *B. vietnamensis* 151–6 could effectively decrease the Cd content of late rice grains in field trials.

4. Discussion

In this study, the gene fragment *orf4108–4109* conferred *E. coli* cells with an increased Cd removal rate, which could explain the reason for high-level Cd absorption in our previously isolated *B. vietnamensis* 151–6. Interestingly, the results of transcriptome sequencing under Cd stress showed that the transcripts of *orf4108* (thiol-disulfide oxidoreductase gene) and *orf4109* (cytochrome C biogenesis protein CcdA gene) increased 156.64- and 130.84-fold, respectively, compared with the control (absence of Cd) (Yu et al., 2020). The fold changes of the two genes were the most obvious among all upregulated genes. Our results suggest that the gene fragment containing *orf4108* and *orf4109* is associated with the Cd response and confers major Cd absorption or binding ability to *B. vietnamensis* 151–6. Some microorganisms have been reported to use metal-binding proteins, such as metallothioneins (MTs), to enhance HM accumulation and/or tolerance (Mejáre and Bülow, 2001). MTs are cysteine-rich metal-binding proteins with low molecular weights (6–7 kDa) that are found in many organisms (Mejáre and Bülow, 2001). However, the only prokaryotic MTs identified to date are present in a few cyanobacterial strains of the genus *Synechococcus*. For example, SmtA in *Synechococcus* PCC 7942 is a typical bacterial MT; it contains four conserved cysteines and two histidines and can bind Cd (II) (Blindauer, 2011). Additionally, other Cd-binding proteins have been identified, such as glycoprotein (Song et al., 2016), Cdae-1 (Mori et al., 2016), and LECBP (Dong et al., 2019). Recently, there have been relatively few reports on bacterial Cd-binding proteins, and it is necessary to identify their novel Cd-binding proteins.

Based on the above, the functions of the Orf4108 and Orf4109 proteins were analyzed. The amino acid sequence of Orf4108 showed homologies to the ResA of *B. subtilis* (Fig. 5). As shown in Fig. 5, both Orf4108 and ResA have the active site motif Cys-Xaa-Yaa-Cys (Erlendsson et al., 2003), which could bind Cd(II). This explains why the heterologous expression of Orf4108 in *E. coli* improved the Cd removal rate of the recombinant strains. Orf4109 was annotated as cytochrome C biogenesis protein CcdA. According to Erlendsson et al. (2003), ResA, which is accompanied by CcdA, is requisite for the reduction of cysteines in the heme-binding site of apocytochrome c. When Cd(II) binds to Orf4108 (ResA), more reducing equivalents are needed in the periplasm, and thus, the cell responds with the upregulation of the *orf4109* gene to produce more CcdA protein. The present study showed that overexpression of the CcdA protein could remove more Cd(II) than the negative strain. Based on the abovementioned analysis, it can be proposed that the Orf4108 and Orf4109 proteins are two Cd-binding proteins for *B. vietnamensis* 151–6.

In addition, field trials were performed to explore the effects of 151–6 on rice growth and Cd accumulation in Cd-polluted soil. Inoculation with *B. vietnamensis* 151–6 effectively decreased the Cd content of the rice grains in late rice, but there was no significant decrease in early rice. In early rice, the average Cd content of the rice grains was approximately 0.12–0.20 mg/kg, while it increased to 0.30–0.58 mg/kg in late rice (Fig. 4), which was in accordance with previous research. Specifically, Liu et al. (2017) reported that the Cd content of rice rachises and grains in early rice in the same paddy field was markedly lower than that of late rice. The most important factors that influenced the great variation in the Cd content in grains between early and late rice were the local climate and humidity. In addition, inoculation with *B. vietnamensis* 151–6 was more effective in decreasing the grain Cd content of high Cd-accumulating rice (Xiangwanxian 12) than that of the low Cd-accumulating rice cultivar (Huazhan) (Fig. 4). These results are consistent with previous studies by other researchers (Liu et al., 2007; Xin et al., 2017).

5. Conclusions

In our study, two potential Cd-binding proteins, Orf4108 (ResA) and Orf4109 (CcdA), of *B. vietnamensis* 151–6 were confirmed to improve the Cd removal rates of the recombinant strains after heterologous expression in *E. coli*. In addition, strain 151–6 can be applied to rice grown in Cd-contaminated soil to promote rice growth and reduce Cd accumulation because the strain has high Cd accumulation ability and potential PGPB properties, including phosphorus dissolution, soluble potassium, and IAA production. The results of the present study highlight the possibility of exploring Cd-absorptive bacteria to protect rice plants in Cd-polluted soil and have expanded our understanding of the Cd-bioremediation mechanism of bacteria.

CRediT authorship contribution statement

XY, JZ, JT, NW conceived and coordinated the study and wrote the paper. XY, JZ, and JT designed, performed and analyzed the experiments. XL and FX provided technical assistance and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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151-6-4108      MKKRKRRLVRTGILLVLISAVVYTLYLNATKDRAIIGEGDMAPDFKLETLDGDTVQLSDY   60
B. subtilis-ResA MKKKRRLFIRTGILLVLICALGYTIYNAVFAGKESISEGSDAPNFVLEDTNGKRIELSDL   60
                 ***::* ::**********.*: **:*  .  .:  *.**. **:* **  :*. ::***

151-6-4108      RGKGVFLNFWGTWCKPCEKEMPYMEKSYQQFKDKGVETLAVNIGESDFLVNKFVEKYDLS  120
B. subtilis-ResA KGKGVFLNFWGTWCEPCKKEFPYMANQYKHFKSQGVEIVAVNVGESKIAVHNFMKSYGVN  120
                 :**************:**:**:*** :.*::**.:*** :***:***.: *::*::.*.:.

151-6-4108      FTFPMDRNRELIDTYGVGPIPTTFLINPEGKVVKVITGSMSQQDIHDYMNMIKPEQ---  176
B. subtilis-ResA FPVVLDTDRQVLDAYDVSPLPTTFLINPEGKVVKVVTGTMTESMIHDYMNLIKPGETSG  179
                 * .  :* :*:::*:*.*.*:*****************:**:*::.  ******:*** :
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Fig. 5. Alignment of the putative ResA amino acid sequence (Orf4108) from *B. vietnamensis* 151–6 versus Res A from *B. subtilis*. Dashes indicate gaps introduced to optimize alignment; asterisk indicates conservative amino acid replacements; semicolons and dots indicate differences; and the black horizontal line indicates a conserved (Cys-Xaa-Yaa-Cys) tetrapeptide.

Data availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.114760.

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