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Cloning, characterization, and expression of cadmium-induced metallothionein-2 gene from earthworm *Pheretima aspergillum* (E. Perrier)

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ABSTRACT. Metallothioneins (MTs) are ubiquitous metal-binding, cysteine-rich proteins, associated with metal accumulation and thus providing protection against toxic heavy metals such as cadmium (Cd). To investigate the mechanisms of enrichment of Cd in the earthworm *Pheretima aspergillum*, we isolated and cloned metallothionein-2 (MT-2) cDNA (538 bp) from *P. aspergillum*, analyzed its sequence, and examined MT-2 transcription levels by relative quantitative real-time PCR under different concentrations of Cd. The sequence of *P. aspergillum* MT-2 cDNA and its putative amino acid sequence were highly similar to sequences from other earthworms. The induction with Cd increased the MT-2 gene transcription level in a dose-dependent manner. In addition, earthworm recombinant MT-2 exhibited high Cd bioaccumulation ability *in vitro*. These results suggested that MT-2 plays an important role in tolerance and accumulation of Cd in *P. aspergillum*.

Key words: *Pheretima aspergillum* (E. Perrier); Metallothionein-2; Cadmium; Cloning

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INTRODUCTION

Along with further development of industrialization and urbanization, metal pollution continues to have detrimental effect on the environment and is increasingly recognized as a hazard for ecosystem dynamics and human health as it can cause disturbances at various levels of biological organization in most species (Liu et al., 2005; Al-Johany and Haffor, 2009). However, many organisms have the capacity to survive in metal-contaminated soils and accumulate heavy metals to some extent (Corp and Morgan, 1991; Zhang et al., 2007; Pellerin and Amiard, 2009; Jung and Lee, 2012) due to their defense mechanisms that protect them from metal stress conditions. Absorbed by these organisms, heavy metals may reach the food chain in significant amounts and pose a threat to human health.

The earthworm *Pheretima aspergillum* (E. Perrier) is not only a common soil animal, but also a traditional Chinese medicinal animal. It has been widely used in clinical practice in Asian countries, especially in China, for its antimicrobial (Li et al., 2011), anti-inflammatory (Balamurugan et al., 2009) and antiasthma (Chu et al., 2007) properties and capability for peripheral nerve regeneration (Chen et al., 2010). Unfortunately, medicinal animals are mainly collected and harvested from the wild habitats and, as previously reported, they demonstrated a strong tolerance to metal pollution through accumulation of heavy metals such as cadmium (Cd) (Li et al., 2005). Administration of *P. aspergillum* specimens with elevated levels of heavy metals would seriously threaten patient's health and life. Understanding the underlying mechanisms of the enrichment of heavy metals in *P. aspergillum* is therefore considered to be of great importance.

Metallothioneins (MTs) are ubiquitously found in a wide variety of living organisms (Kagi and Kojima, 1987). They play a pivotal role in metal detoxification because of their high affinity for metals (Sturzenbaum et al., 2004). In fact, MTs are unusually rich in cysteine residues that coordinate multiple metal ions under physiological conditions (Palmiter, 1998). Multiple isoforms have been reported in many mammals (Vašák and Meloni, 2011) and invertebrates (Hockner et al., 2011), and recent progress in molecular biology allows a more precise characterization of these different forms. In some invertebrates such as *Lumbricus rubellus*, two MT isoforms have been identified and cloned. They were shown to be characteristically rich in cysteine residues and possessed no significant aromatic residues (Sturzenbaum et al., 1998). Although both isoforms could bind equal amounts of Cd, MT-2 was more sensitive in its response to Cd and more stable during proton competition, as shown by chemical analysis of recombination proteins (Sturzenbaum et al., 2001). Since both *P. aspergillum* and *L. rubellus* belong to the earthworm family, it is possible that the former also contains MT-2, thus explaining the enrichment of heavy metals in *P. aspergillum*.

In this study, *P. aspergillum MT-2* gene was isolated. The sequence and expression level of its messenger ribonucleic acid (mRNA) were analyzed in response to Cd stress conditions. We examined its function in tolerance to Cd in *Escherichia coli*, and demonstrate that *MT-2* gene in *P. aspergillum* responds to heavy metals and has high ability for Cd bioaccumulation. Therefore, we conclude that the *MT-2* gene is involved in the enrichment of Cd in *P. aspergillum*, which can be used to develop strategies for reducing excessive levels of heavy metals such as Cd in *P. aspergillum*.

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MATERIAL AND METHODS

Preparation of earthworm

Clitellated *P. aspergillum* earthworms were collected from private fields in southern Guangzhou, China, with a kind permission of the owner and were identified by professor Liwei from Guangzhou University of Chinese Medicine. The earthworms were acclimatized for 2 weeks under laboratory conditions in the soil collected from the collection sites. The soil was analyzed with a Z-2000 flame atomic absorption spectrometer (Hitachi, Tokyo, Japan) to ensure the absence of Cd contamination.

Stress treatment

Dry soil (25 kg) was divided into five wooden boxes (50 x 50 x 50 cm). Cd solution ($CdCl_2$; Tianjin Chemical Industries, Tianjin, China) was added to the soil. Cd was tested at four concentrations (9, 18, 36, and 72 mg/kg). The concentrations were set based on multiples of the content of Cd (0.6 mg/kg) in the secondary standard of Environmental Quality Standard for Soils (GB 15618-1995) in China. Water was used as a control. Moisture content of the soils was adjusted to 21% of the final weight. Later, 20 well-developed clitellated adult earthworms (the average wet weight, 8-13 g) were chosen and cultured in each box. Each treatment was carried out at 20° ± 2°C in constant darkness and was performed for 7 days.

Isolation of RNA and synthesis of cDNA

Total RNA was extracted from the prostate gland and posterior alimentary canal using a TRIpure-reagent (Bioteke, Beijing, China) according to manufacturer instructions. The purity, concentration, and integrity of RNA were measured by monitoring the A_{260}/A_{280} absorbance ratio with a SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA) and agarose gel electrophoresis (1%). For synthesis of cDNA, 2 µg RNA in 20 µL reverse transcription reaction mixture was used with a RevertAid First-Strand cDNA Synthesis Kit (Fermentas UAB, Vilnius, Lithuania) according to the instructions.

Cloning the *MT-2* gene and sequence analysis

MT-2 cDNA was amplified using primers designed from highly conserved amino acid motifs of the MT superfamily as well as the available sequence of *Amynthas aspergillus* MT-2 (accession No. EU560442.1 GI: 189187439). The primers were as follows: TKCCG-forward: 5'-ACTAAATGCTGCTGTGGA-3'; MT-29-forward: 5'-AAGGCGAGAAACTCTTTGTGG CGGA-3'; and MT-566-reverse: 5'-AGCGGCAACAAATGAGAAGC-3'. The 50-µL polymerase chain reaction (PCR) mixture contained 1 µL cDNA, 5 µM of each forward and reverse primers, 5 U Taq DNA polymerase (Fermentas), and 250 µM of each dNTP. The PCR thermal cycler conditions were as follows: initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 40 s and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products of expected size were purified, cloned into a pGEM-T Easy Vector (Promega, Beijing, China), and sequenced at BGI

(Guangzhou, China). The deduced amino acid sequence was aligned and conserved domains were analyzed using ClustalW (Larkin et al., 2007); phylogenetic tree inferred from the MT family nucleotide sequences from earthworm was constructed using the MEGA5 software (Tamura et al., 2011).

Relative quantitative real-time PCR (qPCR)

To assay the MT-2 mRNA expression level in earthworms under different conditions, real-time PCR was performed on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using 2X SYBR® mixture (with ROX) (CWBIO, Beijing, China). PCR protocol consisted of initial denaturation at 95°C for 4 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min, and a dissociation step at 95°C for 15 s. The primers used for qPCR were as follows: MT-forward: 5'-CCAAGGGAGGATAACAAGTG-3', MT-reverse: 5'-CATTTTGCCGAACCACATC-3'; Actin-forward: 5'-GAAACCACTTACAACAGCATCA -3', and Actin-reverse: 5'-GGCGATACCAGGGAACATT-3'. The qPCR data were analyzed by comparative Ct method and relative fold gene expression ($2^{-\Delta\Delta Ct}$) of *MT-2* in stress-treated earthworms compared to control (without treatment) was normalized with internal control actin (GI: 560189341) Ct values.

Expression of MT-2 fusion protein in *E. coli*

The complete coding region of *MT-2* was amplified with special gene primers using pGEM-T-MT-2 as a template. The primers were as follows: open reading frame (ORF)-forward: 5'-CGGAATTCATGTCTGACACTACTAAATGCT-3' (*Eco*RI site underlined) and ORF-reverse: 5'-GGGTTCGAACTACTTGCATGATCCCTTCGCG-3' (*Hin*dIII site underlined). A Dream-Taq (Fermentas) was used in the 50 μL PCR. The PCR products were purified and sequenced to confirm the ORF sequence. Later the purified products were digested with *Eco*RI and *Hin*dIII (Fermentas) and ligated into a PET-32a (+) expression vector (offered from another in-house laboratory). *E. coli* strain BL21 cells (TianGen, Beijing, China) were transformed with the constructed vector and were grown at 37°C in lysogeny broth (LB) containing 100 μg/mL ampicillin. When the optical density of cells at 600 nm (OD_{600}) reached 0.6, the recombinant protein was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 32°C. A noninduced recombinant protein served as the negative control. After induction, the cells were harvested and analyzed with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Western blotting

Western blotting was performed based on the identification of a histidine tag in the fusion protein. Total protein extracts from induced culture expressing MT-2-His fusion protein and from non-induced cells were separated by 12% SDS-PAGE and then electrotransferred to polyvinylidene difluoride membranes (Millipore EMD, Billerica, MA, USA). After blocking in 5% (w/v) nonfat milk for 2 h, the membrane was blotted overnight with an anti-His antibody (1:1000 dilution) (Abmart, Shanghai, China), followed by horseradish peroxidase-labeled goat anti-mouse secondary antibody (Beijing Dingguo Changsheng Biotech, Beijing, China). Finally, the protein bands were detected using an enhanced chemiluminescence reagent.

Bioaccumulation and analysis of heavy metals

Tests for bioaccumulation of heavy metals were conducted using the recombinant *E. coli*. Seed culture (500 µL) was added into a 250-mL flask containing LB-ampicillin broth, cultivated until OD_{600} reached 0.6, and later IPTG and different concentrations of $CdCl_2$ were added to the flask. The final concentrations of $CdCl_2$ ranged from 10 to 500 µM. After 2-h growth, cells were harvested, rinsed three times using growth medium, and dried at 55°C for 24 h. The cell pellets were treated overnight with 5 mL 70% nitric acid and mineralized in an MARS 240/5 microwave oven (CEM Corporations, Matthews, NC, USA). The contents of Cd were determined with a Z-2000 flame atomic absorption spectrometer (Hitachi).

Statistical analysis

All determinations were performed in triplicates and all data are reported as means ± SD. The significance of difference between treatments and control groups was tested by the independent sample *t*-test using SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Cloning and sequence analysis of MT-2

The cDNA sequences of MT-2 from *P. aspergillum* were 538 bp in length and contained a 237-bp ORF (Figure 1), which encoded 79 amino acids with a theoretical isoelectric point of 8.04 and molecular weight of 7.8 kDa. BLAST revealed that *P. aspergillum MT-2* gene shares 93% similarity with *A. aspergillus* (accession No. EU560442.1 GI: 189187439). Phylogenetic analysis of the MT-2 sequences of these and other earthworms confirmed a close relationship of *P. aspergillum* with *A. aspergillus* (Figure 2).

Multiple alignments of amino acid sequences of MT with other earthworms indicated that *P. aspergillum* kept the conserved amino acid sequences of the earthworm family (Figure 3).

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1                                                                   A
2    AGG CGA GAA ACT CTT TGT GGC GGA AAG ATG TCT GAC ACT ACT AAA TGC TGT GGA
                                     M   S   D   T   T   K     C   C   G
56   AAG ACC GCA TGC CCA AGG GAG GAT AAC AAG TGT GCG TGT CCT AAT TGC CAA TGT
      K   T   A   C   P   R   E   D   N   K   C   A   C   P   N     C   Q   C
110  ACA GAC TGT CCC CCG AAC TGC GAC AAG AAT TGT TGT GGC TCA GCA GGA TGT GGT
      T   D   C   P   P   N   C   D   K   N   C   C   G   S   A     G   C   G
164  TCG GCA AAA TGT GGC AAT GTC AAC TGC AAG TGC GGC GCG GAT TGT AAA TGC ACT
      S   A   K   C   G   N   V   N   C   K   C   G   A   D   C     K   C   T
218  GGA GGA CCG GCC TGT GCA ACT GAG TGC GCG AAG GGA TCA TGC AAG TAG CTT GCA
      G   G   P   A   C   A   T   E   C   A   K   G   S   C   K   end
272  GTT GGA ATG ATC TGT GTC AGA TAA CGA CTA TGA GTG GCA GTC TCT AGC AGA AGA
326  ACA TGC ATT TTT TGC CTT TCG GCA GTT CCG CTT GCT TAT TTA CGA TAT ATT TAC
380  ATT TTG TAT CTT GTT TAA GCT CGT CCT AGA TTT GCT TAA TTT AAT TTG TTG AAA
434  ATG AGT TTG CAA AGT TGG ATG TAT TCA ATG GGC TAG CAT TAA TTT CGA GAA CAA
488  TTG ATG ATT TCA AAT TTA AAG TAT TAG TTC TGC TTC TCA TTT GTT GCC GCT
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Figure 1. Nucleotide sequences of cDNA of the *Pheretima aspergillum MT*-2 gene. The deduced amino acid sequence is shown below the nucleotide sequence. cDNA = complementary DNA; MT = metallothionein.

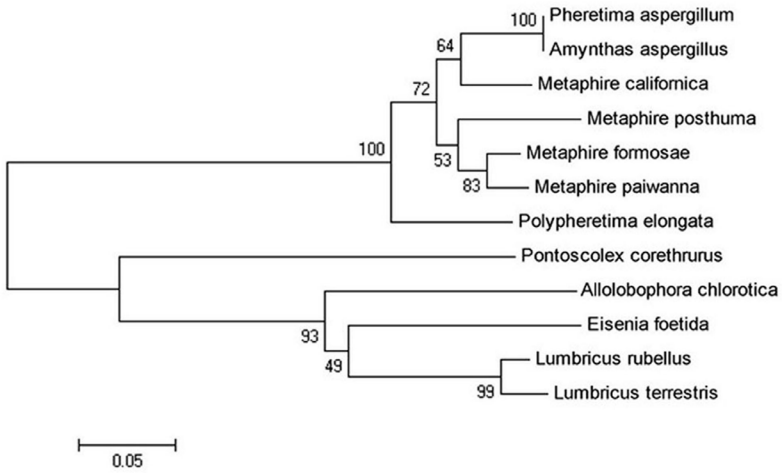

Figure 2. Phylogenetic analysis of MT-2 nucleotide sequences. GenBank accession numbers for the sequences are as follows: *Amynthas aspergillus* (ACD84578.1); *Metaphire californica* (ACD84579.1); *M. posthuma* (ACA60734.1); *M. formosae* (ACD84580.1); *M. paiwanna* (ACD84581.1); *Polypheretima elongata* (ACA60735.1); *Pontoscolex corethrurus* (ACD84582.1); *Allolobophora chlorotica* (ADK62365.1); *Eisenia foetida* (CAA15423.1); *Lumbricus rubellus* (AJ005822.1); *Lumbricus terrestris* (CAA09056.1). MT = metallothionein.

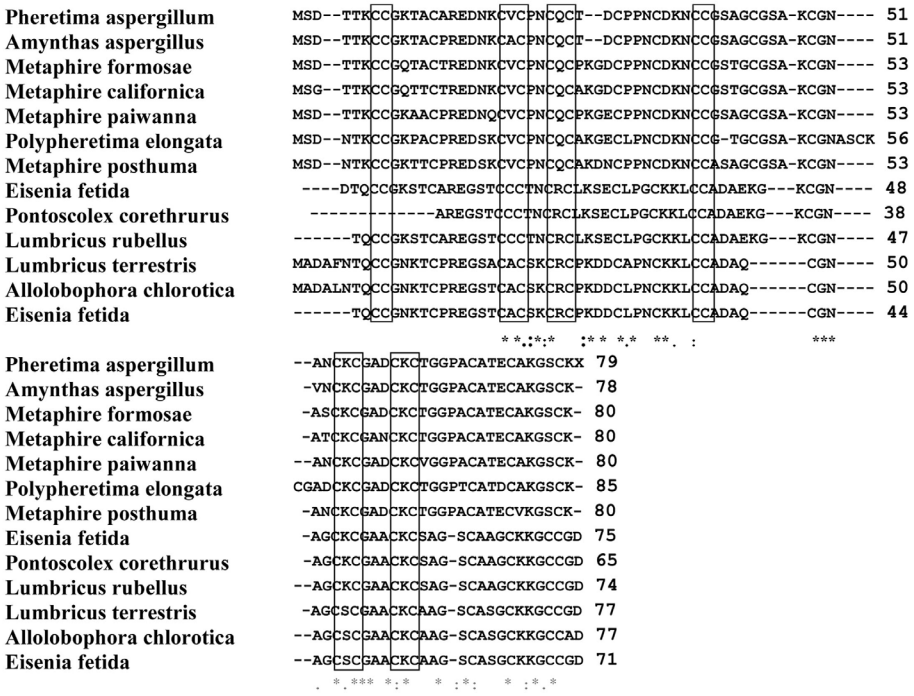

Figure 3. Alignment of the amino acid sequences of the *MT-2* gene from *Pheretima aspergillum* and other earthworm species. Borders indicate functional motifs of the MT-2 protein. For GenBank accession numbers, see legend to Figure 2. MT = metallothionein.

Effects of $CdCl_2$ stress on MT-2 mRNA expression in earthworm *Pheretima aspergillum*

Expression of MT-2 mRNA was measured for a specific time period (7 days) using qPCR. As shown in Figure 4, exposure to higher doses of $CdCl_2$ increased MT-2 transcription levels in *P. aspergillum*, reaching the maximum levels at the highest dose of 72 mg Cd/kg dry soil (18.31-fold greater than the control) (Figure 4).

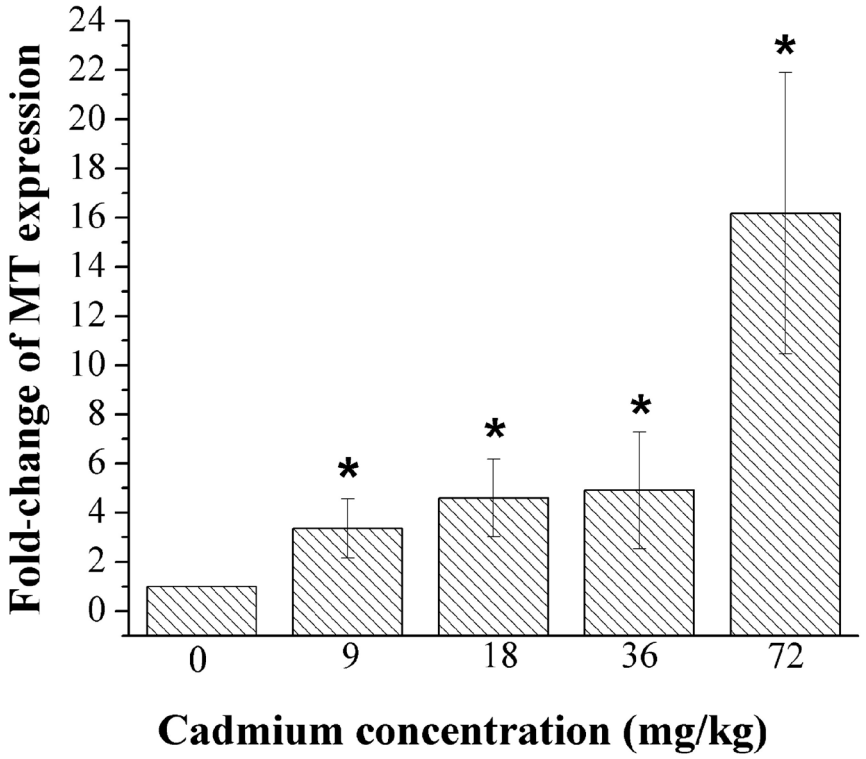

Figure 4. Quantification of the earthworm MT-2 transcription levels. Relative expression levels of MT-2 were normalized by actin. Data are reported as means ± SD. Asterisks denote significant differences. *P < 0.05 *vs* control group. MT = metallothionein; SD = standard deviation.

Expression of MT-2 fusion protein in *E. coli*

As shown in Figure 5A, the molecular weight of the MT-2 fusion protein was in accordance with its predicted molecular weight of 25 kDa under reducing conditions of 5 M IPTG. A band of the same size was not detected under nonreducing conditions. Meanwhile, analysis of the MT-2 fusion protein in the supernatant and lysate revealed a band of 25 kDa, indicating that the MT-2 fusion protein may exist in both soluble and insoluble forms. Moreover, the expression of the MT-2 fusion protein was confirmed by Western blotting using an anti-His antibody (Figure 5B).

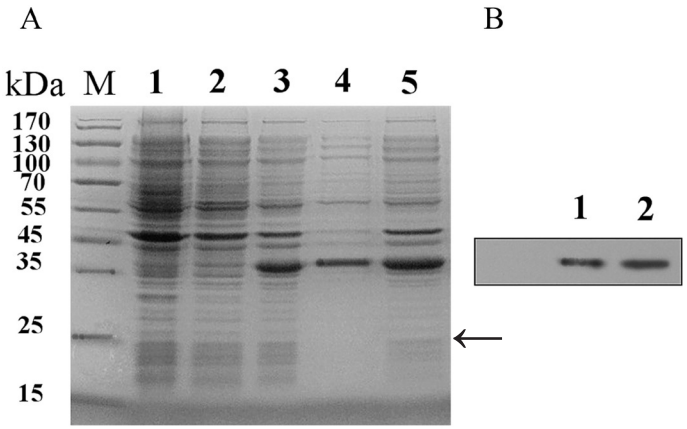

Figure 5. SDS-PAGE and Western blot analysis of the recombinant MT-2 fusion protein. **A.** *Lane 1* = total protein extract of bacterial culture; *lane 2* = total protein extract of noninduced culture; *lane 3* = total protein extract of induced culture expressing the MT-2-His fusion protein; *lane 4* = lysate pellet of the MT-2-His fusion protein; *lane 5* = lysate supernatant of the MT-2 fusion protein; *lane M* = molecular weight marker proteins. The arrow indicates the position of the MT-2 fusion protein (25 kDa). **B.** *Lane 1* = total protein extract of noninduced culture; *lane 2* = total protein extract of induced culture expressing the MT-2-His fusion protein. MT = metallothionein; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Bioaccumulation of the heavy metal

Results from the bioaccumulation test showed that recombinant MT-2 fusion protein had significantly higher capacity to bioaccumulate $CdCl_2$ compared to the control under different concentrations of $CdCl_2$. The accumulated metal ion (Cd^{2+}) content in *E. coli* (pMT) was about 1.9 and 8.8 times greater than in the control *E. coli* (pET) at 10 and 20 µM concentrations of Cd, respectively, but these levels were about 4.4 and 8.3 times higher than in the control at 250 and 500 µM concentrations of Cd, respectively (Figure 6).

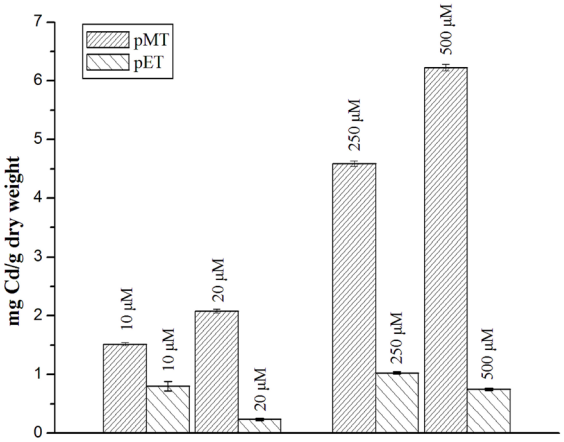

Figure 6. Metal ions accumulated by cells expressing MT-2-His [pMT] with His-expressing cells [pET]. Data are reported as means ± SD.

DISCUSSION

A number of studies have shown that earthworms can survive in heavy metal-contaminated sites (Langdon et al., 1999, 2001; Reinecke, 1999; Reid and Watson, 2005; Arnold et al., 2008) and accumulate heavy metals (van Gestel et al., 1993; Darling and Thomas, 2005; Hobbelen et al., 2006) including Cd, which is a powerful toxic agent to living organisms. The mechanisms involved in heavy metal tolerance may be explained by the presence of metal-binding proteins, which have been proven for many species and probably play a critical role in storing a substantial body burden of metals (Dallinger, 1996; Fraysse et al., 2006). The presence of metal-binding proteins in oligochaetes was first described in 1980 (Suzuki et al., 1980), and later they were successfully isolated from *L. rubellus*, Megascoleidae earthworms, *Metaphire posthuma*, *Polypheretima elongata*, and other types of earthworms, but not from *Pheretima aspergillum*. Thus, molecular cloning presents a useful tool in understanding molecular characteristics of MT-2 in *P. aspergillum*. In the present study, a 538-kb cDNA of the *P. aspergillum MT-2* gene was isolated. Phylogenetic analysis and multiple alignments revealed that the cloned *MT-2* gene from *P. aspergillum* belongs to the MT family, and it is highly similar to *A. aspergillus*, suggesting that these two species may have originated from a common ancestor. In addition, MT-2 in *P. aspergillum* was found to contain conserved cysteine residues and functional motifs including four Cys-X-Cys and two Cys-Cys, which play an essential role in chelating heavy metals (Figure 3). Furthermore, of the 79 amino acids that constitute MT-2 protein, 19 (24%) were cysteines. This percentage is similar to the range of 23-33% seen in other MTs (Liang et al., 2009). These features confirm that this cDNA sequence indeed codes for MT-2 in *P. aspergillum*.

It is well established that the induction of MT is stimulated by a heavy metal stress (Amiard et al., 2006). To evaluate the effect of exposure to heavy metals, the expression level of the *MT*-2 gene from *P. aspergillum* was quantified by qPCR technique under treatment with different concentrations of Cd on a specific time scale (7 days). As shown in Figure 4, Cd stress resulted in a significant up-regulation in the expression of MT-2 mRNA in the earthworm in a dose-dependent manner. This finding corroborates previous reports on *M. posthuma*, *Polypheretima elongata*, *Pontoscolex corethrurus*, and *L. rubellus* (Spurgeon et al., 2004; Liang et al., 2011). Besides, strong induction was observed during the exposure to high concentration of $CdCl_2$ (72 mg/kg), whereas all tested earthworms were viable without apparent lethal outcome, including the soils treated with 100 mg/kg Cd (data not shown). This level exceeded the maximum permitted content of Cd by >300 times in earthworms that are used as a medicine according to "Chinese Pharmacopoeia". These results indicate that production of MT-2 may allow tolerance of earthworm to metal contaminants after exposure to Cd.

To explore whether MT-2 permits the enrichment with heavy metals and helps in adaptation to environment, biochemical properties of the recombinant earthworm MT-2 proteins were analyzed. The MT-2 recombinant protein was expressed in *E. coli* BL21 cells using a PET-32a (+) expression vector. To obtain the optimum conditions for expressing the recombinant protein, different time intervals, IPTG concentrations, and temperatures were investigated. The optimum conditions were observed in the treatment with 5 M IPTG at 32°C for 2 h (Figure 5A). Western blot analysis showed that the MT-2 fusion protein corresponded to its predicted molecular weight (Figure 5B), thus demonstrating that the recombinant MT-2 protein was successfully expressed in *E. coli* BL21 cells. *E. coli* strains were successfully grown in the presence of low and high concentrations of $CdCl_2$ (Figure 6). The metal-binding capac-

ity of the recombinant MT-2 fusion protein allowed growth under increasing concentration of $CdCl_2$ in either case, which suggest that the high capacity for storage of Cd is a characteristic feature of MTs. Similar evidence for absorbability of Cd has been reported in recombinant MT of sheep (Sauge-Merle et al., 2012), humans (Ma et al., 2011), etc. For instance, recombinant *E. coli* strain TB1 (TB1-MT) of mammalian MT assimilated about 0.93 mg Cd/g dry cells (8.33 μmol Cd/g dry cells) (Sauge-Merle et al., 2012). In this study, *E. coli* BL21 (pMT) adsorbed 2.08 mg Cd when exposed to 20 μM Cd, which was about 2.2 times higher than TB1. No doubt MT-2 is vital for bioaccumulation of Cd in earthworms in a polluted environment.

Previous studies mainly focused on earthworm's ability to bioaccumulate metals for monitoring metal pollution (Zhang et al., 2009) and considered MTs as a good biomarker to assess metal pollution in soil (Calisi et al., 2011; Maity et al., 2011). However, for *P. aspergillum*, which is a species widely used in traditional Chinese medicine, bioaccumulation properties of the species severely affect the safety of the clinical medication. Therefore, the ability of enrichment with heavy metals of *P. aspergillum* warrants careful consideration. In this study, the *MT-2* gene was cloned and characterized, and the MT-2 recombinant protein was expressed in *E. coli* BL21 cells to understand the underlying mechanisms of the enrichment with heavy metals in *P. aspergillum*. It is speculated that MT-2 data presented in this study will be valuable for future exploration of the ways to reduce the accumulation of heavy metals in *P. aspergillum*.

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