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Accumulation and tolerance to cadmium heavy metal ions and induction of 14-3-3 gene expression in response to cadmium exposure in *Coprinus atramentarius*

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ABSTRACT

Cadmium (Cd), one of the most toxic heavy-metal pollutants, has a strong and irreversible tendency to accumulate. Bioremediation is a promising technology to remedy and control heavy metal pollutants because of its low cost and ability to recycle heavy metals. *Coprinus atramentarius* is recognized as being able to accumulate heavy metal ions. In this work, *C. atramentarius* is cultivated on a solid medium containing Cd^{2+} ions to analyze its ability to tolerate different concentrations of the heavy metal ion. It is found that the growth of *C. atramentarius* is not significantly inhibited when the concentration of Cd^{2+} is less than 0.6 mg L^{-1}. The accumulation capacity of *C. atramentarius* at different Cd^{2+} concentrations also was determined. The results show that 76% of the Cd^{2+} present can be accumulated even when the concentration of the Cd^{2+} is 1 mg L^{-1}. The different proteins of *C. atramentarius* exposed to Cd^{2+} were further analyzed using gel electrophoresis. A 14-3-3 protein was identified and shown to be significantly up-regulated. In a further study, a full-length 14-3-3 gene was cloned containing a 759 bp open reading frame encoding a polypeptide consisting of 252 amino acids and 3 introns. The gene expression work also showed that the 14-3-3 was significantly induced, and showed coordinated patterns of expression, with Cd^{2+} exposure.

1. Introduction

The environmental problems associated with urban growth in the last few decades have continued to increase correspondingly. Rapid urbanization and industrialization creates increased demand for transportation and energy as human activity increases, e.g. heavier vehicle traffic and industrial activity (Grigoratos et al., 2014). Pollution due to heavy metal escape (Pb, Zn, Cr, Ni, Cd, etc.) is one of the most important problems in the urban environment (Sawidis et al., 2011). The importance of such pollutants needs to be viewed in light of the currently incessant urban growth and the adverse health effects with which they are linked (Kampa and Castanas, 2008; Kelepertzis, 2014). Human exposure to Cd, for example, may be linked to cancer formation, kidney and bone damage, and hematuria (Satarug et al., 2010). Since 1912, numerous studies have reported on Cd contamination of soil, food crops, water, and sediments around the world, implying serious potential health risks to humans (Zhuang et al., 2009; Robson et al., 2014).

There is clearly a need for new technologies to clean the environment to safe limits. Suitable techniques to use must be easy to handle, be cost-effective, and be feasible. A range of technologies is already available for remediation of metal contamination, e.g. physical, chemical, soil amendment, and biological methods, and also phytoremediation, biotechnology, and nanotechnology approaches (Singh et al., 2015). Compared to conventional techniques, the main advantages of bioremediation are reduced cost and reduced exposure risk for cleanup personnel. In addition, bioremediation eliminates waste permanently and eliminates long-term liability. It can also be coupled with physical or chemical treatment technologies. Furthermore, it is a non-invasive technique and leaves the ecosystem intact (Vidali 2001).

Mycoremediation, a form of bioremediation, involves using fungi to degrade or sequester contaminants in the environment. Dudhane et al. (2012) reported that mycorrhizal fungi secrete glomalin (a glycoprotein) to stabilize aluminum in soil as well as in the roots of *Gmelina* plants. Taştan et al. (2010) investigated

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the heavy-metal bioaccumulation potential of *Aspergillus versicolor*. They found this species to have optimal effects at pH values of 6 for 50 mg L^{-1} Cr(VI) and Ni(II), and 5 for 50 mg L^{-1} Cu(II) ions. Furthermore, the removal yields were 99.89, 30.05, and 29.06%, respectively. Further to this, Ramasamy et al. (2011) found that a different fungal strain (*Aspergillus fumigates*) is able to remove Pb(II) ions from aqueous solution. In this case, a maximum adsorption of 85.41% was realized.

Coprinus atramentarius is a wild edible mushroom that is widespread in China. It is the second best known ink cap and member of the genus *Coprinus* after *Coprinus comatus* (Huang et al., 1992). In order to analyze the mycoremediation potential of *C. atramentarius*, we investigated the tolerance and removal efficiency of *C. atramentarius* with respect to the heavy metal ion Cd^{2+}. A differential protein expression analysis identified a significantly up-regulated 14-3-3 protein which we further cloned. We also determined the cadmium-induced gene expression of the 14-3-3 gene.

2. Materials and methods

2.1. C. atramentarius isolation and growth conditions

The *C. atramentarius* strain used was kindly provided by Dr. Liao at the Chongqing Academy of Agricultural Sciences. The *C. atramentarius* was maintained in a modified potato dextrose culture medium (200 g L^{-1} potato extract, 20 g L^{-1} dextrose, 20 g L^{-1} corn starch, 0.5 g L^{-1} $MgSO_4$, 1 g L^{-1} KH_2PO_4, 2 g L^{-1} peptone, pH 7.0–7.5 — a solid medium containing 2% agar).

2.2. Growth response and tolerance of C. atramentarius to Cd^{2+}

The $CdCl_2$ was added to solid medium to final concentrations of 0, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 3 and 5 mg L^{-1}. The *C. atramentarius* was inoculated onto the plates at 25 °C and three replicate experiments carried out at each concentration. Mycelial growth was measured after nine days (the colony diameters were recorded every day and the corresponding growth curves constructed).

2.3. The accumulation capacity of C. atramentarius towards Cd^{2+}

The $CdCl_2$ was added to liquid medium to final concentrations of 0.5, 0.8, and 1 mg L^{-1}. The *C. atramentarius* was inoculated in a constant temperature room (25 °C) using a rotary shaker (180 rpm). After nine days, 7–8 ml of the liquid cultures were harvested every other day. These were centrifuged at 12,000 rpm, 4 °C for 10 min. The Cd^{2+} concentrations in the supernatant liquids were then measured using an atomic absorption spectrophotometer.

2.4. Protein extraction and SDS-PAGE procedure

The fungal cultures were filtered through 0.22 mm nitrocellulose filters and the supernatant further concentrated by freeze drying. The total protein of the fungal mycelia was extracted using an acetone/trichloroacetic acid (TCA) precipitation method. Briefly, the tissues were first ground into a fine powder in liquid nitrogen using a cold mortar and pestle. Then, 0.5 g of the fungal mycelia was transferred to a centrifuge tube and 5 ml of pre-chilled acetone containing 10% w/v TCA added. This was kept at −20 °C overnight. The samples were centrifuged at 8000*g* for 10 min at 4 °C and then the pellets were washed twice using 80% acetone. The pellets were dried at room temperature to remove the acetone. The dried samples were stored at −80 °C. The samples were then dissolved in a 7 M urea solution.

SDS–PAGE was carried out using the Leamilli method (Laemmli, 1970). Polyacrylamide gel (12% w/v) was used in the Bio-Rad (Mini-PROTEAN) electrophoresis system at 150 V for 1.5 h and 20 μg of solubilized protein sample was loaded in each lane. After electrophoresis, the gel was stained with silver stain.

2.5. Protein identification and database search

Spots of interest were cut out of the gels and transferred to microcentrifuge tubes. The gel particles were washed twice with deionized water, and 50 μl decolorized solution (50 mM NH_4HCO_3/CH_3CN (1:1)) was added. The samples were then vortexed for 20 min at 37 °C, and the decolorization step repeated until the blue color had disappeared. To shrink the gel particles, we added 50 μl of acetonitrile until the particles became white. The gel particles were then dried in a vacuum centrifuge for 10 min and mixed with 20 μl of a solution containing 10 mM DTT in 25 mM NH_4HCO_3. This was kept for 1 h at 56 °C to reduce the proteins. The gels were then dried at ambient temperature in a vacuum centrifuge for 30 min. They were then incubated in 20 μl alkylation solution (50 mM iodoacetamide in 25 mM NH_4HCO_3) for 45 min in the dark. Several washes were then performed in succession: 25 mM NH_4HCO_3 (2 × 10 min), 25 mM NH_4HCO_3 in 50% CH_3CN (2 × 10 min), and CH_3CN (10 min). The gel particles were completely dried in a vacuum centrifuge for 10 min and enzymatically digested overnight at 37 °C using 5 ng ml^{-1} trypsin.

The digested peptides were then extracted into 0.1% TFA in 50% CH_3CN and analyzed using an Ultraflex TOF/TOF (Bruker, Germany). A Maldi matrix of α-Cyano-4-hydroxycinnamic acid (4 mg/ml, 70% ACN: 30% ddH_2O, containing 0.1% TFA) was used. Parent-ion masses were measured in delayed extraction mode with an accelerating voltage of 20 kV. The autolytic fragments of trypsin acted as internal calibrants. Spectra were acquired in positive reflectron mode (20 kV) and collected within the mass range of 700–4200 Da. The mass fingerprint spectra were then processed using flexAnalysis (v2.0) software. Database searches were performed using the Mascot search engine (http://www.matrixscience.com). The following search parameters were applied using the NCBI protein database: a mass tolerance of 0.2 Da and one missed cleavage was allowed; carboxyamidomethylation of cysteine was specified as a fixed modification; and oxidation of methionine was allowed as a variable modification.

2.6. Molecular cloning and expression analysis of the 14-3-3 gene

Total RNA was isolated from *C. atramentarius* using RNAiso Plus (TAKARA, Dalian, China). The first strand cDNA synthesis reaction was catalyzed by M-MLV (Promega) using the QT primer. The cDNA was used as a template to amplify the core region of the 14-3-3 with degenerated primers (14-3-3 core F/R) whose designs are based on the conserved cDNA sequence regions 14-3-3 from other fungi. 3′ RACE was carried out following the Scotto-Lavino et al. (2007) protocol. The 5′ direction fragments were amplified from *C. atramentarius* genomic DNA using hiTAIL-PCR.

To analyze the expression of 14-3-3 on exposure to Cd^{2+}, semiquantitative RT-PCR was conducted using actin1 gene as endogenous control. The part coding sequence of *C. atramentarius* actin1 was cloned with the degenerated primers (Actin1 Core F/R) (Supplemental Dataset 1). The primers in the study are shown in Table S1.

3. Results

3.1. Effect of Cd^{2+} on the growth phase of C. atramentarius

Mushrooms interact both physiologically and morphologically with heavy metals. Some heavy metals have important biological roles in the fungal metabolism but some are considered toxic at