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ORIGINAL PAPER

Intracellular sequestration of manganese and phosphorus in a metal-resistant fungus *Cladosporium cladosporioides* from deep-sea sediment

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Abstract A heavy metal resistant fungus was isolated from the sediment of Pacific Ocean, and identified to be *Cladosporium cladosporioides*. It grew normally in a medium containing 60 mM Mn^{2+} and could endure 1,200 mM as the highest concentration tested. Quantification analysis confirmed a high accumulation of Mn which was 58 mg/g in dried biomass. Under transmission electron microscope, many intracellular crystals were observed in the cytoplasm of the hypha cells grown in a Mn-rich medium, and varied from a few nanometers to 200 nm in length. Energy dispersive X-ray (EDX) analysis showed that the crystals were composed of manganese and phosphorus in atomic ratio of 1.6:1 (Mn/P). Further, factors which might influence the resistance of this fungus were investigated. As a result, its high resistance to Mn^{2+} was found dependent on the presence of Mg^{2+}, and could be further enhanced by phosphate. However, the effect of phosphate was not observed without the presence of Mg^{2+}. In addition, the resistance was also influenced by pH of the medium, which was lost above pH 8. This is the first report on a fungus which showed a hyper resistance to manganese by forming a large quantity of intracellular Mn/P crystals.

Keywords *Cladosporium cladosporioides* · Heavy metal resistance · Intracellular sequestration · Manganese · Phosphorus

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Introduction

Manganese is an important constituent of soil and aquatic sediments. It is also an essential element to all living organisms, working as a coenzyme and involved in many biochemical processes. However, excessive manganese will inevitably disorder cell metabolism. Its toxicity can be indicated by the minimum inhibitory concentration (MIC), which is defined as the lowest concentration to completely inhibit cell growth. In the reported microorganisms, the MIC of Mn^{2+} is usually below 30 mM, for example, 20 mM for *Escherichia coli*, 24 mM for a metal tolerant *Bacillus circulans* (strain EB1; Yilmaz 2003), and 10 mM for *Saccharomyces cerevisiae* (Yang et al. 2005).

On the other hand, microorganisms have evolved mechanisms to tolerate the toxic level of different heavy metals, by active efflux, sequestration or enzymatic detoxication, etc. (Bruins et al. 2000; Fredrickson et al. 2000; Joho et al. 1995; Nies 1999; Rouch et al. 1995; Silver and Phong 1996; Silver et al. 1989; Wakatsuki 1995). Among them, active efflux is the most ubiquitous and effective way to resist metal toxicity, which is conducted by the action of specific transporters located on cell membrane (Silver et al. 1989; Nies and Silver 1995; Arnesano et al. 2002).

Sequestration is another important way in some fungi and bacteria to deal with heavy metal toxicity. Two kinds of sequestration are usually found, intracellular or extracellular. Extracellular sequestration is a direct binding to cell wall or with secreted chelating molecules. For examples, *S. cerevisiae* sequesters Ni(II) externally by excreting large quantities of glutathione (Murata et al. 1985), and *Klebsiella aerogenes* excretes sulfur to precipitate Cd(II) ion (Aiking et al. 1982;

Scott and Palmer 1990). Inside the cells of both fungi and bacteria, metallothioneins are found responsible for binding, sequestering and buffering excess intracellular divalent ions, such as Cu^{2+}, Zn^{2+}, Cd^{2+} (Lerch 1980; Olafson et al. 1988; Robinson et al. 2001). In addition, intracellular sequestration may need the ion active influx as the first step.

Recently, intracellular phosphate was found to be related with heavy metal resistance in some bacteria. Aiking et al. (1984) first reported that accumulation of phosphate in cytoplasm was involved in the tolerance of Cd in *K. aerogenes*. Keasling and Hupf (1996) also found that an enhanced hydrolysis of intracellular polyphosphate helped to protect the host cells against Cd toxicity. More recently, it has been discovered that uranyl nitrate could trigger the hydrolysis of intracellular polyphosphate as well as the deposition of uranyl phosphate at the cell membrane in *Pseudomonas aeruginosa* (Renninger et al. 2004).

In the case of manganese, extracellular oxidation is detected in both bacterium and fungus. Bacterial Mn^{2+} oxidization by spores of marine *Bacillus* sp. strain SG-1 has been extensively investigated (Mandernack et al. 1995; Francis and Tebo 1999, 2002). Manganese oxide was formed outside of the spore by putative Mn^{2+} oxidases (Tebo et al. 2004, 2005). Recently, two soil fungi were reported able to oxidize Mn^{2+} to manganese oxide outside the cell (Miyata et al. 2004; Thompson et al. 2005).

Little is known about intracellular sequestration of manganese, except a report about a subsurface bacterium that could form tiny Mn-rich granules when either birnessite or pyrolusite served as the electron acceptor during bacterial growth (Glasauer et al. 2004). However, this is a quite unclear process and has not been reported to be related with metal resistance. In this study, we isolated a fungus from the deep-sea sediment of Pacific Ocean, which demonstrated high resistance to Mn^{2+} with a MIC of more than 1,200 mM. What is more, it could accumulate manganese and phosphorus as a crystalline complex in cytoplasm.

Materials and methods

Deep-sea sediment

Sediment of Pacific Ocean was collected with a multicore sampler during the cruise of DY105-13 of R/V ''Hai-Yang Si Hao'' in May 2002, at the site of WP02-3 (E147° 8.9206′, N12° 59.9060′) with a depth of 4,480 m. Sediment in the column was processed in a bio-clean working bench, and put in a sterile sampling cup with a cap and sealed with parafilm. Samples were stored at 4°C prior to use.

Culture media

HLB medium contains 3% NaCl, 0.5% yeast extracts and 1% Tryptone, prepared with distilled water (pH 7.3); SWPDA medium was prepared with natural seawater from the Pacific deep-sea, containing 2% sucrose and 1.2% potato extracts (product of Beijing Shuangxuan Microbial Cultural Medium Factory, China) (pH 7.2). About 1.6% agar was added to make a solid medium. APDA medium was an artificial seawater medium which contains 3% NaCl, 0.7% $MgSO_4$, 2% sucrose and 1.2% potato extracts, prepared with tap-water (pH 7.2).

Isolation of heavy metal resistant microbes

To isolate heavy metal resistant microbes from the sediment samples, the enrichment experiment was conducted by suspending 5 g of the sediment in 100 ml HLB medium supplemented with 5 mM $CdSO_4$. After 14 days of incubation at 20°C, tiny black flocs appeared in the medium. By spreading the flocs on an SWPDA plate, a fungus was obtained and named PSf-1. It has been deposited in Marine Culture Collection Center of China, and numbered as 3A00002. Its resistance to other heavy metals including Mn^{2+} was further tested by inoculating spore-water suspension to metal-containing SWPDA medium in a tube, and incubated in a shaker at 100 rpm, 20°C, for 5 days.

$MnSO_4$, $CoCl_2$, $HgCl_2$, $AgNO_3$, $Pb(NO_3)_2$, $CuSO_4$, $NiCl_3$, $CdSO_4$, $NaAsO_2$, $ZnSO_4$ and $SrCl_2$ were used to prepare heavy metal containing medium. Metal concentrations were set from 0 to 24 mM to test the tolerance for each of the heavy metals, except $MnSO_4$ and $SrCl_2$, the highest concentration used was 1,200 and 120 mM, respectively. The resistance was reflected by biomass, which was harvested and washed with double distilled water and then freeze-dried to a constant weight. All treatments were set in triplicate unless specified.

Growth conditions

Optimal growth temperature was examined with a liquid medium of SWPDA in a rotary shaker at: 4, 8, 12, 18, 24, 28, 32, 38 and 48°C. The optimal growth pH was tested in the same medium with pH adjusted before autoclaving from 2 to 11, cultivated at 25°C. The optimal salinity for growth was first tested from 0, 5, 10, 15, 20, 25, 30 to 35% NaCl in APDA medium, then examined from 0 to 5% with a gradient of 0.5%.

Examining the factors influencing PSf-1 resistance to Mn^{2+}

The medium APDA added with or without $MgSO_4$ was used to test the effects of Mg^{2+} on the resistance to Mn^{2+}. In pH treatments, liquid SWPDA was adjusted to different pH values after being added with 100 mM $MnSO_4$. To examine the effect of phosphorus, sterilized 0.2 M PBS buffer (pH 7.0) was used to provide a concentration gradient of phosphate from 0.1 to 5 mM in the liquid medium. All tests were carried out in duplicate, the cultivation conditions used were 25°C, 150 rpm with 10 ml of medium in each tube.

Microscopy

The morphological characteristics of the fungus were examined with a phase contrast microscope. Transmission electron microscope (TEM) was used to visualize the accumulation of manganese in the fungus.

The fungus was cultivated on an HLB plate containing 15 mM $MnSO_4$. Hyphae were fixed with 2.5% glutaraldehyde, rinsed with 0.1 M PBS (pH 7.2) for 15 min and further dehydrated with a series of concentrations of ethanol from 40% to the anhydrous. The dehydrated hyphae were embedded in a water soluble resin Lowicry K4M kit according to the instructions provided by the manufacturer (Polysciences Inc), ultrasectioned at 600 nm, and sticked to a copper grid without film coating. Ultrathin sections were directly observed without electron staining, at 100 KV with a JEOL TEM (model JEM-100CX).

To observe the subcellular structure, sections were stained with 2% uranyl acetate in 70% ethanol and observed with a JEOL TEM (model JEM-2010). The fungus in this experiment was grown in liquid SWPDA medium, which contained 20 mM $MnSO_4$. The hyphae were collected after 7 days of incubation and were processed for TEM examination as described earlier.

Energy dispersive X-ray (EDX) analysis

For EDX analysis, sections were directly observed under a JEOL TEM (model JEM-2010) without staining and analyzed with the attached X-ray detector. The observed intracellular crystals were photographed digitally (Gatan, model 794) and their EDX spectra were collected using the area scanning function, and the chemical composition of the targeted crystal was read by the attached software.

Heavy metal quantification

The fungus was cultivated in liquid SWPDA containing $MnSO_4$ of 1, 2, 4, 10 and 20 mM, respectively, at 20°C in a shaker. The biomass was harvested after 7 days of growth, and dried to a constant weight at 100°C in a desiccation oven. An atom absorption spectrometer, Spectr AA20 (Varian tech), was used to quantify manganese in the dried biomass and in the culture supernatant. The supernatant was diluted with 1.5% HNO_3 in a volumetric flask and applied to the apparatus directly. The dried biomass was digested with concentrated nitric acid at room temperature for 5 h, and then heated on a hot plate to complete dissolving, and then diluted with double distilled water in a volumetric flask.

Phylogenetic analysis

Genomic DNA of the fungus strain PSf-1 was extracted using CTAB according to Saghai-Maroof et al. (1984). The cell wall of the hyphae was digested at 37°C with 1% snailase before cell lysis.

The fungus-specific primers TR1 5′-GTTTCTAG GACCGCCGTA-3′ and 5′-CTCAAACTTCCATC GACTTG-3′ were synthesized according to Bock et al. (1994) and used to amplify a 581-bp fragment within the gene coding for the small ribosomal subunit (18 S rDNA) of fungi. Primers ITS5: 5′-GGAAGTAAAA GTCGTAACAAGG-3′ and ITS4: 5′-TCCTCCGC TTATTGATATGC-3′ were used to amplify the ITS1-5.8S-ITS2 rDNA fragment. They were designed according to White et al. (1990). PCR steps in both 18S and ITS analyses were: initial denaturation at 95°C for 4 min, followed by 30 cycles of 1 min at 95°C, 1 min at 53°C, and 2 min at 72°C and a final extension at 72°C for 12 min. PCR products were purified and recovered using the UNIQ-5 PCR Product Purification Kit (Sangon). Then, the recovered amplified fragments were inserted into pUCm-T vector (Sangon). The ligated products were transformed into *E. coli* DH5α cells. The inserted fragments were sequenced with a model 377 automated DNA sequencer using a BigDye Terminators Cycle Sequencing kit (Applied Biosystems). The sequence data were blasted on NCBI, and the related sequences from GeneBank were analyzed with DNAMAN (version 5.1, Lynnon Biosoft). Phylogenetic trees of 18S rDNA, and ITS (ITS1-5.8S-ITS2) sequences were constructed by the neighbor-joining method (Saitou and Nei 1987) using the programs of DNAMAN. Bootstrapping analysis was used to evaluate the tree topology of the neighbor-joining data by performance of 10,000 trials. The 18S

rDNA of *Penicillium chrysogenum* strain UPSC 2020 (AF548087) was used as an outgroup reference.

Results

Isolation of a heavy metal resistant fungus

After 2 weeks of incubation with the sediment of Pacific Ocean at the site of E147° 8.9206′, N12° 59.9060′, a fungus appeared as black pellets in the enrichment with HLB medium containing 5 mM Cd^{2+}. The culture liquid was diluted and spread on plate to get a single colony. The fungus were numbered as PSf-1.

The spores and conidiophores of the fungus were observed with characteristics of the genus *Cladosporium* (http://www.mycology.adelaide.edu.au/Fungal_Descriptions/Hyphomycetes_(dematiaceous)/Cladosporium/). On plate medium, the colony grew dark greenish. The conidiophores were short, and most spores were round, while some were in a lemon shape (Fig. 1a). The vegetative hyphae were straight with apparent septae (Fig. 1b). In liquid SWPDA medium, the hyphae were narrow and straight, and could produce free cells, resembling holoarthric conidia (Fig. 1c).

Interestingly, cell morphology changed when the fungus grew on an HLB plate of 15 mM Mn^{2+} (this is a toxic level without the presence of Mg^{2+}, as shown later) (Fig. 1d). The cells became about 2–4 times as big as those in Fig. 1a–c, and became opaque and rough (Fig. 1d). Some black granules were observed in the cells (Fig. 1d). This was consistent with the following results observed with TEM (Fig. 2).

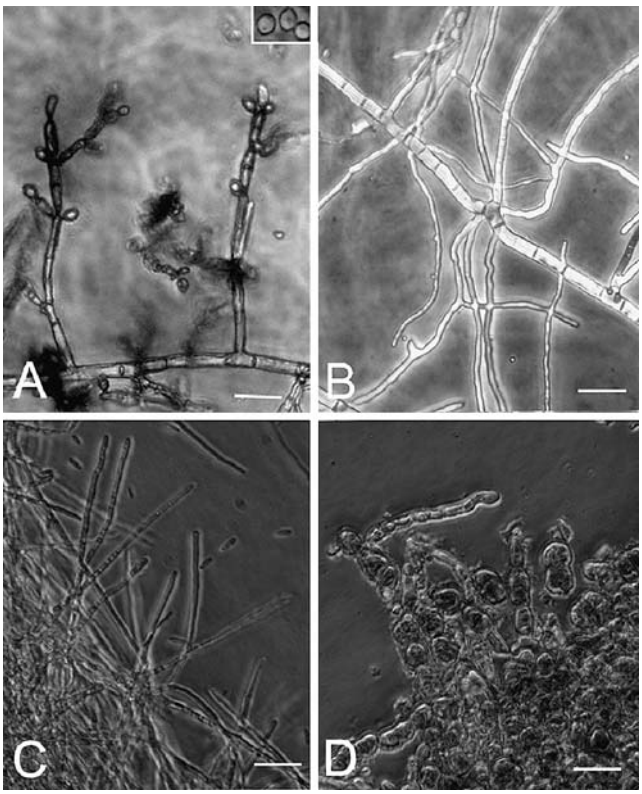

Fig. 1 PSf-1 observed under a phase contrast microscope (bar = 10 μm). **a** PSf-1 conidiophore phialide (corner: conidia). **b** PSf-1 hyphae; both **a** and **b** from PDA (3% NaCl) plate. **c** Hyphae grown in liquid medium. **d** Hyphae grown on HLB (NaCl 3%) plate containing 15 mM Mn^{2+}

Physiological characteristics

Optimal growth temperature was tested at 4, 8, 12, 18, 24, 28, 32, 38 and 48°C in liquid SWPDA. The results showed that the PSf-1 grew slowly at 4°C and could not grow at 38°C; the best growth was observed between 18 and 28°C, which is higher than that of the deep-sea environment (usually 1–2°C). Growth pH was tested from pH 2–11 in liquid SWPDA; the results showed that PSf-1 can grow from pH 3 to pH 9, and showed better growth in acid than in alkaline range (Fig. 4). The best growth occurred from pH 5 to 7, as determined by dry weights. However, pH 3, 4 and 8 also brought about a nice growth, especially at pH 3. But no growth was observed at pH 2 and 10 (Fig. 4).

Optimal growth salinity was tested in liquid APDA with sodium chloride concentration ranging from 0 to 35%. At 25% salinity, only slight growth was observed and no growth was observed above 30%. In the first round test, the best growth was found to be 5%. In the second round test from 0 to 5%, the best growth occurred from 3 to 4%, followed by 1.5–2% and then 0–1%. Therefore, the optimal salinity was the natural salinity of seawater.

Resistance to manganese and other heavy metals

$MnSO_4$ was added to the liquid SWPDA medium at a series of concentrations to test the resistance capability. No inhibition was observed on the growth of PSf-1 below 60 mM of manganese, and partial inhibition was observed in 100 mM Mn^{2+}(referring to Fig. 3). It still showed a slight growth at 1,200 mM of Mn^{2+}, which is the highest concentration used in this study.

The resistance of PSf-1 to other heavy metals was also tested, including $CoCl_2$, $HgCl_2$, $AgNO_3$, $Pb(NO_3)_2$, $CuSO_4$, $NiCl_3$, $CdSO_4$, $NaAsO_2$, $ZnSO_4$ and $SrCl_2$. No growth was observed in 1 mM Hg^{2+}, and only slight growth in 1 mM Ag^{+} and Pb^{2+}. The MIC to other metals was tentatively determined to be: Cu^{2+} 8 mM, Ni^{3+} 8 mM, Cd^{2+} 16 mM, Co^{2+} 16 mM, AsO_2^{-}

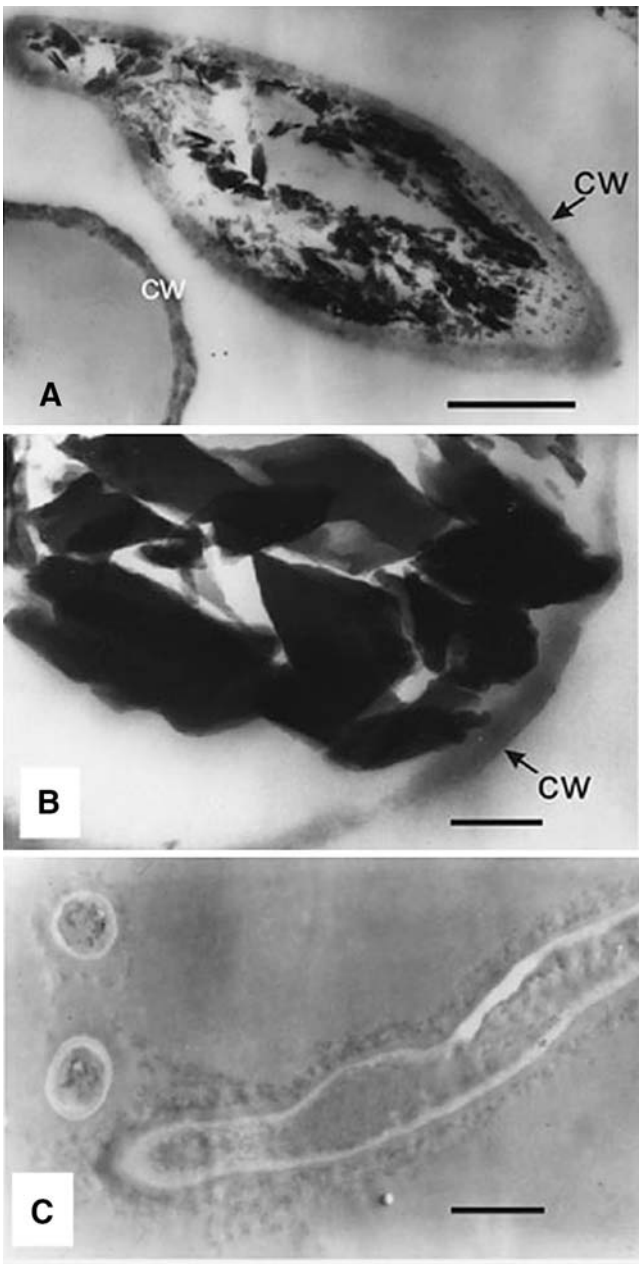

Fig. 2 Manganese accumulation in PSf-1 observed under TEM without electron staining. Many crystals of varied size occurred in the cytoplasm of Mn^{2+} treated cells under TEM, and showed a high electron density. **a** Intracellular manganese deposits in PSf-1, hyphae were from an HLB plate of 15 mM Mn, bar = 1 μm; *CW* cell wall. **b** An enlarged view of intracellular crystals in **a**, bar: 200 nm. **c** Mn^{2+} minus control, bar = 300 nm

20 mM, Zn^{2+} 24 mM, and Sr^{2+} 100 mM, respectively. The minimum concentrations in which PSf-1 showed no growth inhibition were: Cu^{2+} 1 mM, Ni^{3+} 1 mM, Cd^{2+} 1 mM, Co^{2+} 2 mM, AsO_2^- 2 mM, Zn^{2+} 2 mM, and Sr^{2+} 30 mM. Thus, among the tested heavy metals, PSf-1 was most sensitive to Hg^{2+}, $Ag^{+}$and Pb^{2+}, followed by Cu^{2+}/Ni^{3+}, Cd^{2+}, Co^{2+}, AsO_2^-, Zn^{2+}, and Sr^{2+}; it is most resistant to Mn^{2+}.

Intracellular crystals of manganese and phosphorus

The accumulation of manganese in the fungus was observed with TEM. The hyphae were grown on an HLB plate containing 15 mM Mn^{2+}. As mentioned earlier, the cells were expanded and filled with black granules as observed under the light microscope (Fig. 1d). For TEM, the hyphae were fixed with glutaraldehyde (no postfixation), embedded, ultrathin-sectioned and directly observed under TEM at 100 KV without electron staining. Many large crystals of high electron density were observed in the cytoplasm (Fig. 2a). They varied from 200 nm to only a few nanometers in length, and not constrained to a certain area in the cell, and no heavy metal was adsorbed on cell wall as well (Fig. 2b). In an enlarged view, crystals showed a sharp edge and an irregular shape (Fig. 2b). In contrast, no such crystals were observed in the cytoplasm of hyphae grown in medium without Mn addition, and the cells looked slenderer than those Mn treated cells (Fig. 2c).

The effect of Mg^{2+} on Mn^{2+} toxicity

The tolerance of PSf-1 to Mn^{2+} was substantially reduced in an APDA medium without Mg. For example, when challenged with 12.5 mM Mn^{2+}, the fungus showed a serious growth inhibition and resulted in only 33% dried biomass of the control without Mn^{2+} addition. When the concentration of Mn^{2+} was raised to 25 mM, PSf-1 grew very slightly (0.30 mg/ml), and at 50 mM the growth was almost completely inhibited (Fig. 3a).

However, in the APDA medium, which contains 0.7% $MgSO_4$, the resistance of PSf-1 was significantly enhanced (Fig. 3b). It grew well in a high concentration of Mn^{2+}. For example, in a medium containing 200 mM Mn^{2+}, PSf-1 produced 2.2 mg/ml dry biomass; and at 50 mM Mn^{2+}, nearly no inhibition was observed (dry weight, 7.0 mg/ml); while at 25 mM, the dry weight reached 7.6 mg/ml, which was even higher than that of the no Mn control (7.1 mg/ml).

Resistance was highest in the treatments with natural seawater prepared medium (Fig. 3c). There was no inhibition at low Mn^{2+}concentrations (below 50 mM in this test); Similarly with the case in APDA medium, the biomass was increased when Mn^{2+} was added, e.g., at 25 mM Mn^{2+}, it was increased by 11% of the control. These results confirmed that Mg^{2+} played

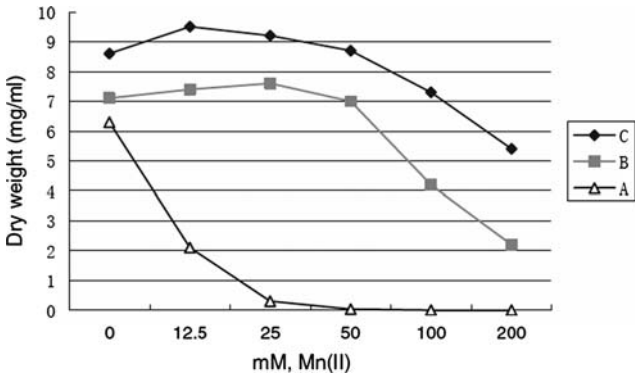

Fig. 3 Effect of Mg^{2+} on the toxicity of Mn^{2+} to PSf-1: **a** growing in APDA without adding Mg^{2+}; **b** growing in APDA containing 0.7% $MgSO_4$; **c** growing in SWPDA. Treatments in **a**, **b** and **c** contained Mn^{2+} from 0 to 200 mM, and set in triplicate. The dry weight was the average of 3 days biomass per mililiter

an important role in Mn^{2+} resistance of PSf-1. On the other hand, increasing Mg^{2+} from 20 to 320 mM by a series of concentration did not show a corresponding enhanced protection against 100 mM Mn^{2+} (data not shown).

The effects of pH and phosphate on Mn^{2+} toxicity

To detect the effects of pH on the Mn^{2+} toxicity to PSf-1, the pH of liquid SWPDA was adjusted to a range of values from 2 to 10 after autoclaving. Each treatment was paralleled with an Mn^{2+} non-adding control to reflect the toxicity of Mn^{2+}. The dried biomasses in both cases were measured (Fig. 4). Results showed that the resistance to Mn^{2+} (100 mM) was significantly reduced in alkaline pH, as only a very slight growth happened at pH 8, and no growth was observed above pH 9. However, in acid pH range from pH 3 to pH 7, the influence of pH change was not obvious, the reduction in dry weight caused by Mn^{2+} depression varied from 3 to 24%. Noticeably, the fungus seemed to grow best morphologically at pH 3, either with or without Mn, but the actual dry weight was not the highest.

The effect of phosphate on Mn^{2+} toxicity was tested by adding 0.2 M PBS (pH 7.0) to SWPDA and APDA (with or without additional Mg^{2+}). In SWPDA medium containing 100 mM Mn^{2+}, phosphate at low concentration (from 0.1 to 0.5 mM) was beneficial to cell growth, which resulted in an increase of dry weight by 11–36%, comparing to the control cells. However, excess quantity of phosphate (4–8 mM) failed to enhance the growth, but reduced the biomass instead. Protective effect of phosphate was more obvious in APDA than in SWPDA medium. From 12.5 to 200 mM of Mn^{2+}, all treatments showed a positive effect of phosphate (Fig. 5). When 1 mM phosphate was present, normal growth was recovered from the inhibition by 100 mM of Mn^{2+}. In the case of 200 mM Mn^{2+}, P addition doubled the biomass of the non-adding control. However, in the medium of APDA without adding Mg^{2+}, such an effect of phosphate (0.5 mM) was not detected (data not shown).

Absorption of manganese

The absorption of manganese during growth of the culture was examined with atom absorption spectrometry. The fungus was cultivated in liquid SWPDA containing 1–20 mM of $MnSO_4$. The hyphae were collected and dried after 7 days of growth; both the biomass and the culture supernatant were digested

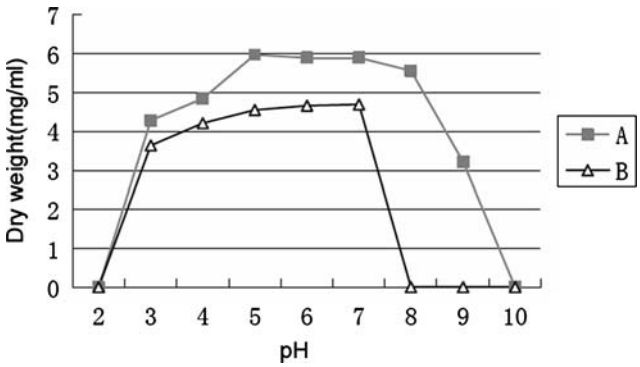

Fig. 4 The effects of pH on PSf-1growing and its Mn^{2+} resistance. **a** The biomass variation of PSf-1 growing in SWPDA from pH 2 to 10 and without adding Mn^{2+}; **b** Growing in SWPDA of 100 mM Mn^{2+}, pH from 2 to 10. The dry weight was the biomass of 2 days cultivation. All treatments were set in duplicate and repeated for two times

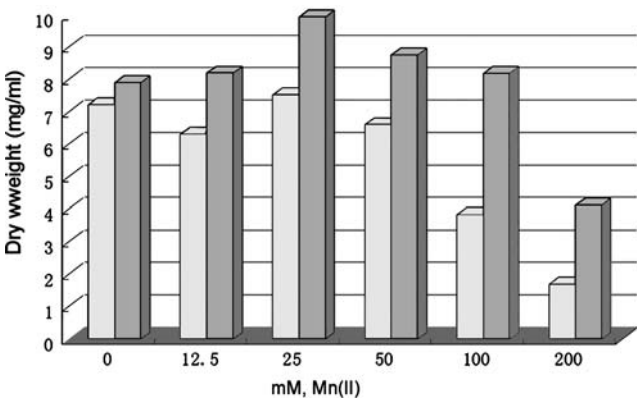

Fig. 5 The effects of phosphate on Mn^{2+} resistance of PSf-1. The medium is APDA containing 12.5 to 200 mM Mn^{2+}; the control is no Mn adding. *Left* no phosphate was added. *Right* phosphate was added to 1 mM. All treatments were set in triplicate

with 1.5% HNO_3 before apparatus analysis. The results showed that 58 mg/g of manganese accumulated in dried biomass treated with 20 mM Mn^{2+}, while 10.1 mM Mn^{2+} was left in the culture. In other treatments of 1, 2, 4, and 10 mM Mn^{2+}, 0.53, 0.98, 2.14, 5.02 mM remained in the culture, respectively. Thus, in all cases, about half of the manganese was absorbed.

EDX analysis results

Energy dispersive X-ray spectroscopy analysis was performed with EDX detector equipped on TEM. Hyphae were the same samples with those in absorption analysis, which were grown in liquid SWPDA containing 20 mM manganese. Sample was processed in the same way as for TEM, sections were not electron stained, firstly observed under TEM, and then analyzed with EDX.

As a result, many crystals were observed inside the cell (Fig. 6a), but they were smaller and not as many as those in Fig. 2. EDX analyzed area was enlarged in Fig. 5b. It showed a very strong signal of manganese (Fig. 6c), as well as a strong signal of phosphorus (Fig. 6c); the two elements exhibited an atomic ratio of 1.63:1 (Mn/P). In contrast, neither Mn nor P was detected in the area of cell wall. In the spectrum profiles, the signal of copper was derived from copper grid, not from samples (Fig. 6d). The signal of Zn (Fig. 6c) indicated the accumulation of Zn^{2+}, which might occur simultaneously with Mn^{2+} from the medium as a divalent cation.

Phylogenesis of the fungus

Blastn results of a partial sequence of 18S rDNA of Psf-1 (a 581 bp) demonstrated that this isolate had a high homology to several fungal species of different genera. For example, it was 99% homologous to *Cladosporium cladosporioides* (AF548070) and *Raciborskiomyces longisetosum* (AY016351) and 98% homologous to *Aureobasidium pullulans* (AY141179), *Phyllobaeis erythrella* (AF491848) and *Capnobotryella renispora* (AY220614). Phylogenetical analysis showed that PSf-1 was mostly related to *C. cladosporioides* (Fig. 7a). Blastn with the ITS sequence (ITS1-5.8S-ITS2) of PSf-1 showed that most of the highly homologous species belonged to *Cladosporium* (up to 97% homology). Other closely related strains were *Davidiella tassiana* strain ATCC26362 (AY361982), which actually also belongs to *Cladosporium.* An exception is *Lacazia loboi* (AF035674), which was detected in dolphin tissue, also showed 97% homology with ITS of PSf-1. Phylogenetic analysis again revealed that PSf-1 was mostly related to *C. cladosporioides* strain ATCC64726 with 97% identity (Fig. 7b). These phylogenetical results, together with morphological characterization, suggested that PSf-1 was an isolate of *C. cladosporioides*.

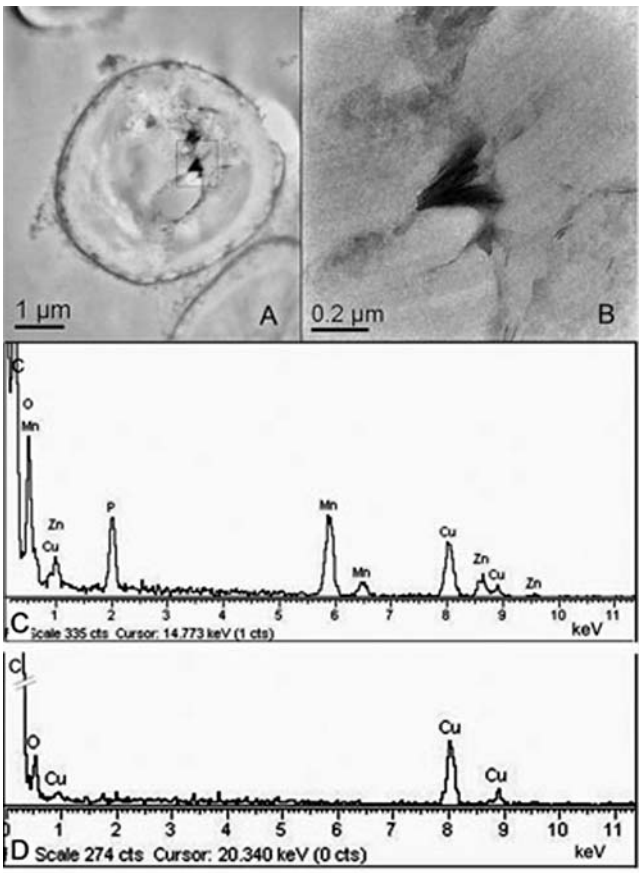

Fig. 6 EDX analysis of intracellular crystals. **a** PSf-1 cultivated in SWPDA liquid medium containing 20 mM $MnSO_4$. Sections were not electron stained. **b** EDX analyzed area, the enlarged part of **a**. **c** EDX spectrum of the crystal in **b**, to show the strong signal of Mn and P. **d** EDX spectrum of the cell wall in **a**, to show the signal background

The sequences were deposited in EMBL Nucleotide Sequence Database, with an accession no. AM286196 for 18S rDNA, and AM286197 for ITS1-5.8S-ITS2 of PSf-1.

Discussion

A heavy metal resistant fungus, identified to be *C. cladosporioides*, was isolated from deep-sea sediment of Pacific Ocean. It showed a high resistance to manganese as well as to many other heavy metals. Before this, two isolates of *C. cladosporioides* have been reported to show preferential sorption of gold, silver, copper or cadmium ions by cell wall of dead biomass

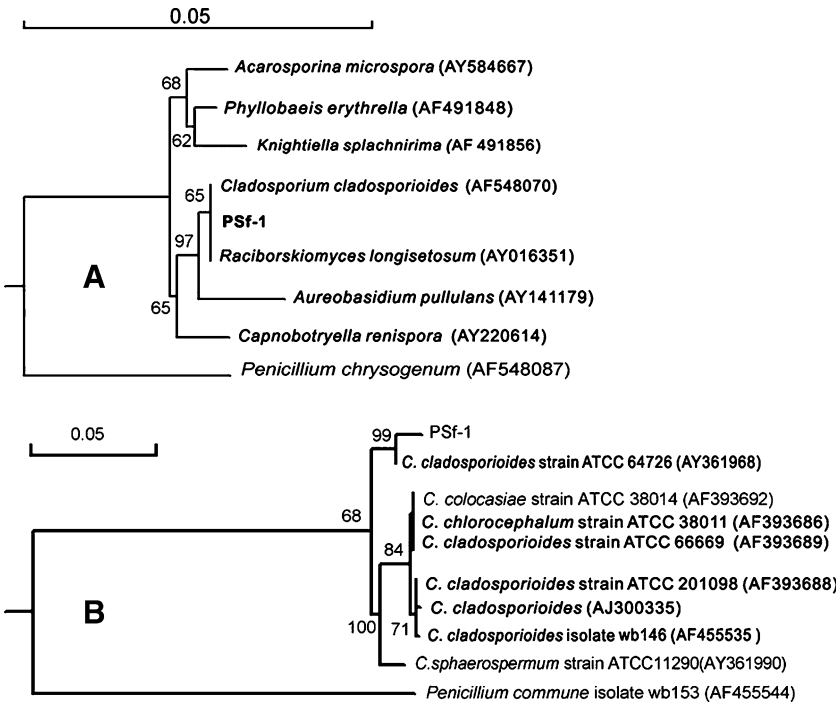

Fig. 7 Phylogenetic analysis of PSf-1. **a** Rooted phylogenetic tree of 18S rDNA sequences constructed by the neighbor-joining method to show the relation of PSf-1 with other taxa, with *Penicillium chrysogenum* (AF54808) as an outgroup. The bootstrapping trial number was 10,000. **b** Rooted phylogenetic tree of ITS1–ITS2 and 5.8S RNA gene. The tree was constructed with *Penicillium commune* (AF455544) as an outgroup. The bootstrapping trial number was 10,000

(Pethkar et al. 2001). However, PSf-1 in this report can sequester the excessive manganese in the cytoplasm by an unknown mechanism, and shows no detectable adsorption on cell wall.

By now, no other case has been found that Mn intracellular sequestration is related with the metal resistance according to our knowledge. We speculate that Mn^{2+} influx is the first step of intracellular accumulation. Further study is needed to illustrate the process. In Mn^{2+} transport studies, *S. cerevisiae* is the most extensively investigated species. The yeast can accumulate Mn^{2+} mediated by transporters located on cellular membrane; however, the influx is just maintained at the level to meet the cell's physiological needs, and only 0.3 mg/g of manganese can be accumulated in dry matter (Stehlik-Tomas et al. 2004). Besides, the yeast showed no resistance to Mn^{2+}, its half lethal concentration of Mn^{2+} was no more than 1 mM (Gadd and Laurence 1996). Remarkably, in this study, manganese was accumulated as high as 58 mg/g in the dried biomass of PSf-1, and no growth inhibition was observed in 60 mM Mn^{2+}. Thus, a special transport system must be involved in Mn bioaccumulation in this fungus.

Further, it is interesting to find that the tolerance of PSf-1 to Mn^{2+} was dependent on Mg^{2+}. Without adding magnesium in medium, the resistance to Mn^{2+} was substantially reduced (Fig. 3). The protective effect of Mg^{2+} has been also observed in both *S. cerevisiae* (Blackwell et al. 1997) and an arbuscular mycorrhizal fungus *Glomus claroideum* BEG23 (Malcova et al. 2002). Besides, phosphorus was also involved in manganese accumulation in PSf-1, not only as an important constituent of the intracellular crystals (Fig. 2), but also as one of the factors to enhance the resistance of PSf-1 (Fig. 5). However, phosphate failed to enhance Mn^{2+} tolerance in absence of Mg^{2+}. These results indicated that Mg^{2+} might act as a coenzyme of transporters for phosphate and/or Mn^{2+}, and pH might have changed the activity of postulated transporters and lowered the Mn resistance in an alkaline environment.

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