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NOTE

Biosorption and bioaccumulation of lead by *Penicillium* sp. Psf-2 isolated from the deep sea sediment of the Pacific Ocean

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Abstract A lead resistant fungus was isolated from the Pacific sediment. It was associated with *Penicillium* according to its partial sequences of 18S and ITS. The fungus could grow in the presence of 24 mM $Pb(NO_3)_2$ in a liquid medium, and no growth inhibition was observed at 4 mM and below. When growing in the presence of 4 mM $Pb(NO_3)_2$, the fungus accumulated a large amount of lead granules in the cell, as well as adsorbed on the outer layer of cell wall, as observed under a transmission electron microscope. The intracellular lead deposited either in the vicinity of the cytoplasm membrane or in the vacuoles, and also could aggregate into large particles in the cytoplasm. However, lead was not adsorbed on the thick inner wall of the fungus. Energy dispersive X-ray spectroscopy analysis showed that these granules or particles mainly consisted of lead, and other elements could hardly be detected. Selected area electron diffraction analysis showed that there were regular crystalline lattices in the lead precipitates, indicating that they were actually in the form of crystals to some extent. Therefore, both intracellular bioaccumulation and extracellular biosorption had contributed to the high resistance of this fungus to lead. These results suggest that this fungus can be used in biotreatment as a lead trapper.

Keywords *Penicillium* · Lead · Heavy metal resistance · Bioaccumulation · Biosorption

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Introduction

Lead is one of the nonessential heavy metals, a group which also include Ag, Cd, Au and Hg. It is detrimental to all organisms even at trivial levels, potentially due to ligand interactions with large biomolecules, such as by inactivation of a series of enzymes. On the other hand, both bacteria and fungi are found to be resistant to lead, although the cases are not as frequently reported as for Cr, Cd and Hg-resistant microorganisms. *Ralstonia metallidurans* CH34 is the most deeply investigated metal resistant bacterium; its resistance to lead is mediated by a P-type ATPase which can transport lead out of the cell (Diels et al. 1989; Borremans et al. 2001; Mergeay et al. 2003). Other bacteria such as *Arthrobacter* sp. (Trajanovska, et al., 1997), *Pseudomonas marginalis*, *Bacillus megaterium* (Roane, 1999), *Staphylococcus aureus* and *Citrobacter freundii* (Levinson, et al, 1996; Levinson and Mahler, 1998) are also reported to be lead resistant, but were not so thoroughly investigated as *R. metallidurans* CH34.

Compared to bacteria, lead resistant fungi are much less investigated (Bååth 1989; Ngu et al. 1998; Dursun et al. 2003; Zucconi et al. 2003). Fungal biosorption of heavy metals are mostly investigated with dead biomass by far. However, bioaccumulation with living cells is recognized to have irreplaceable advantages in the removal of heavy metals, especially from organic sewage (Malik 2004). Therefore, highly resistant microorganisms and a better understanding of metal-microbe interactions will facilitate the application of this technology.

Deep sea is the least explored area on the earth, where microbial resources are still mostly in the dark. In this report, we isolated a fungus of high resistance to lead (II) from the deep sea sediment of the West Pacific Ocean. Its resistance to lead was attributed to both extracellular

adsorption and intracellular accumulation of this metal, which showed a strict preference for certain subcellular structures.

Isolation of lead resistant microorganisms

The lead resistant fungus was isolated from the deep sea sediment of the west Pacific at the site of E147° 8.9206′, N 12° 59.9060′, with water depth of 4,480 m. Lead resistant microorganisms were enriched with 2 mM $Pb(NO_3)_2$ as selective pressure in HLB medium. HLB contains 3% NaCl, 0.5% yeast extracts and 1% Tryptone, prepared with distilled water (pH 7.3). Phylogenetic analyses of ITS1-5.8S-ITS2 and 18S of this fungus were performed as described previously (Shao and Sun 2007). The Blastn results of 18S partial sequence (564 bp) showed that it had 99% homology with species of different genera, such as *Penicillium*, *Eupenicillium*, *Thysanophora*, *Talaromyces* and so on. Certainly, most fungi on the list belonged to *Penicillium*. The Blastn result of ITS (599 bp) confirmed that this fungus belonged to *Penicillium*. However, it cannot be affiliated with any specific species because there are several species of *Penicillium* of 89% identity with the ITS sequence of Psf-2. They are *Penicillium commune*, *P. chrysogenum*, *P. camemberti*, *P. aurantiovirens*, and so on. Therefore, this fungus is possibly a new species of *Penicillium*. It was tentatively named *Penicillium* sp. Psf-2, and deposited in the Marine Culture Collection Center of China with accession number MCCC 3A00002. The sequences of 18S and ITS1-5.8S-ITS2 of this fungus have been deposited in GenBank with accession numbers of EF660440 and EF660439, respectively.

Resistance of this fungus to lead

The resistance of Psf-2 against lead was tested in the range from 0 to 24 mM $Pb(NO_3)_2$, specifically at 2, 4, 8 and 16 mM. The fungus was inoculated with a suspension of conidiophores and cultivated at 28°C, 150 rpm. The medium is liquid PDA which contains 2% sucrose and 1.2% potato extracts (product of Beijing Shuangxuan Microbial Cultural Medium Factory, China), and supplemented with 3% NaCl and 0.18% $MgSO_4$, and adjusted to pH 4 before autoclaving. Lead was added separately after sterilization of the medium. However, lead precipitation occurred at high concentrations of $Pb(NO_3)_2$; the precipitates were visible in the medium above 8 mM. But with the fungus growing, the precipitates finally disappeared from the medium. This may be due to the solubilizing effects of some organic chelators or acids produced by the fungus. Nonetheless, the actual concentration of free lead ions in the medium should be lower than that added to a certain degree.

Although we are not sure about the exact concentration of free Pb ions in the medium, the high resistance of Psf-2 is definite. As a result, Psf-2 can grow even in 24 mM Pb(II), which was the highest Pb concentration tested in this report and it grew normally, i.e., no growth inhibition was observed at 4 mM and below. However, growth was obviously inhibited at 8 mM, which resulted in about 70% biomass reduction of the no Pb control.

It is known that lead is usually toxic to normal fungi even at low concentrations. Such as in baker's yeast, *Saccharomyces cerevisiae*, growth was affected at 10 mg/l of lead (II) (about 0.05 mM). Additions of 50 mg/l lead (II) resulted in a 60% reduction in biomass yields of yeast (Donmez and Aksu 1999). The lead tolerance of *Pseudomonas vesicularis*, *Bacillus cereus*, *Staphylococcus* sp. and *Streptomyces* sp., was reported from approximately 1 to 5 mM (Hasnain et al. 1993; Zanardini et al. 1997). Among the reported lead tolerant fungi, *Paecilomyces lilacinus* is probably of the highest resistance, and it can grow in medium containing up to 1,434 mg/l Pb (about 7 mM) (Zucconi et al. 2003). A marine fungus *Corollospora lacera* (ATCC 34603) isolated from sand grains from Bermuda has also been investigated for its lead resistance; about 41% biomass reduction was caused by 250 mg/l lead (about 1.2 mM) after 15 days of incubation in liquid medium (Taboski et al. 2005). However, in this report, even after discounting the insoluble part of Pb in the treatment of 16 or 24 mM, the concentration of Pb (II) would be no less than 8 mM. The resistance of Psf-2 to lead is remarkable. Additionally, its resistance could be further promoted if other conditions were optimized.

But, how does this fungus resist high concentrations of lead? Several kinds of interactions of fungi with toxic metals have been found: extracellular precipitation, binding to the cell wall, intracellular binding to proteins, vacuolar compartmentation and metal transformation (Gadd 1993; Baldrian 2003). In a previous report, we found a fungus from the same deep sea sample, which could grow in medium containing 1,200 mM Mn(II) by accumulating a large quantity of manganese intracellularly (Shao and Sun 2007).

Mechanism of resistance of Psf-2 to lead

To observe the mechanism of this fungus, the hyphae grown in 4 mM $Pb(NO_3)_2$ were picked out from the culture and subjected to sample processing for transmission electron microscopy (TEM, model JEM-1230EX). The samples were fixed only with 2.5% glutaraldehyde, dehydrated and embedded in resin as described previously. Fixation with

osmic acid was not used to avoid the possible interference of Os. Ultra thin sections of about 600 nm thick were prepared and examined under TEM directly without the regular double staining. However, in order to locate where Pb deposited, part of the ultrathin sections were stained with uranyl acetate.

Results of TEM revealed that lead precipitated both inside and outside of the cell. The precipitation outside the cell seemed to be the first step in a cell's battle with Pb, as in some cells no Pb accumulated in the cytoplasm, but rather just precipitated on the cell wall which occurred only in young cells. Interestingly, the crust of

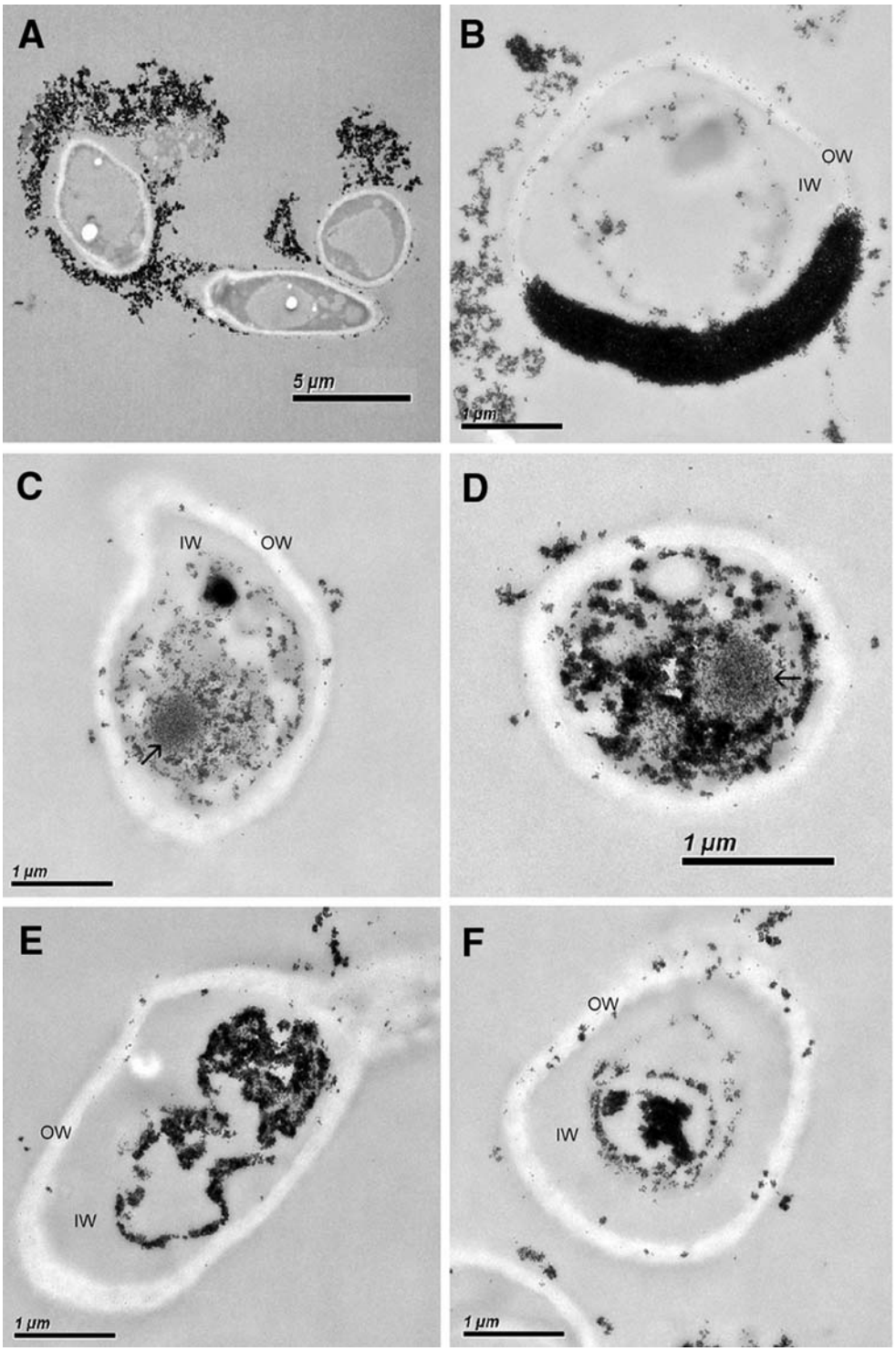

Fig. 1 TEM photographs of Psf-2 grown in the presence of 4 mM Pb (II) (no Os fixation and without electron staining, observed at 80 KV). **a**, **b** Show the cell wall biosorption of lead; **a** a loose binding to the outer layer of the cell wall (*OW*), but no lead had been accumulated in the cell at this point; **b** a tight and thick binding of lead to the outer layer. **c**, **d** Show the intracellular bioaccumulation of lead in the cytoplasm and along the cell membrane, arrow head pointed to the spherical area containing many fine lead granules; **e**, **f** show the intracellular bioaccumulation in vacuoles but not on the inner wall (*IW*). IW was not observed in **a** and **d**

lead on the cell surface could be peeled off in the process of sample preparation (Fig. 1a). On the whole, nearly all cells had surface deposits, but only some had a deposit layer of Pb as thick as those in Fig. 1a, b; actually, most cells had Pb particles on walls as shown in Fig. 1c–f.

Analytically, two layers of the cell wall were observed in most cells. The outer layer grew light, thin and floppy while the inner is dark, thick and dense. Interestingly, lead precipitation mainly occurred on the outer layer. The biosorption occurred to only part of cell surface in some cells (Figs. 1b, 2d) and sometimes around the whole cell (not

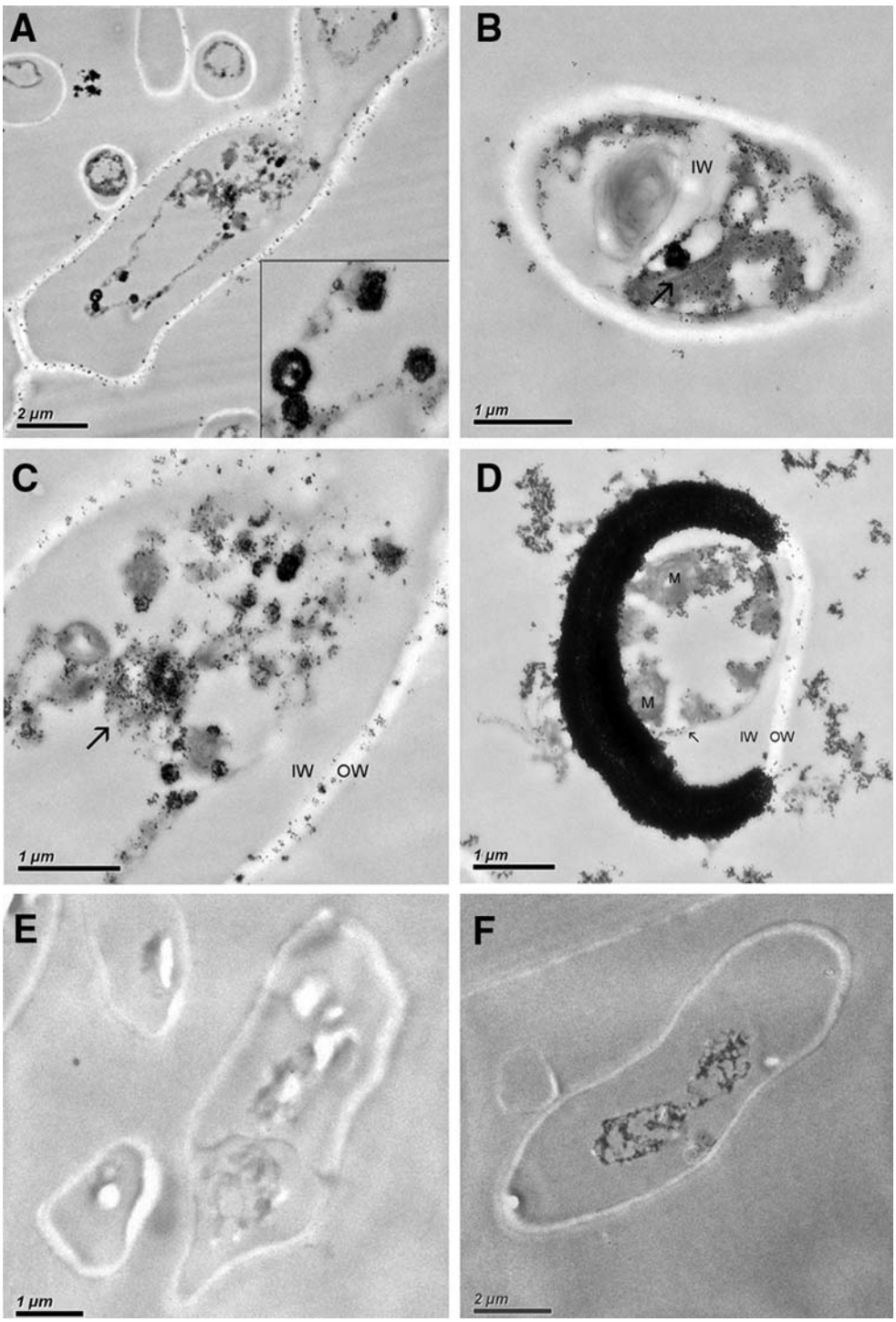

Fig. 2 Ultrastructure of Psf-2 grown in the presence of 4 mM Pb (II) (the same sample as in Fig. 1, observed at 80 KV). **a**–**f** Were U-stained sections except **e**; **a**, **b** whole cells to show the interaction of lead with membranes. **a** Lead deposited in vacuoles and in some vesicles which were enlarged in **c** and at the corner of **a**, and the aggregation of fine granules into large particles can be seen (*arrow head*); **b** showing lead accumulation in the endoplasmic reticulum (*arrow head*). **d** Shows the mitochondria and lead on the cell membrane (*arrow head*) and cell wall. **e**, **f** The controls, which grew in medium without the addition of lead, the section of **e** is without staining while **f** is U stained

shown). Therefore, this layer should be the first defense of Psf-2 against lead toxicity.

Apart from cell wall biosorption, intracellular accumulation occurred in Psf-2 as well. Most cells observed by TEM had intracellular deposits of Pb in the form of irregular granules of about 15–100 nm (Fig. 1c, d), and both the protoplasm and vacuoles were the sites of accumulation. The cell membrane seemed to be closely related with Pb enrichment (Fig. 1c–f), as Pb particles dotted on the inner surface of the cell membrane. Conspicuously, in the cytoplasm of some cells, many fine granules of Pb aggregated into a ball (Figs. 1c, d, 2c, arrow head pointed). In vacuoles, the granules were seldom located at the center, but rather near the inner side of the vacuole membrane, and were usually larger than those in the cytoplasm (Figs. 1f, 2b).

In U stained sections, we can see clearly that granules of lead localized mainly in the vicinity of membranes (Fig. 2a), and that they can be aggregated into large particles in vacuoles (Fig. 2b, c). Some localized at the channel of the endoplasmic reticulum as well (Fig. 2b, arrow head), but scarcely in the mitochondria (Fig. 2c, d). Certainly, some lead granules were not membrane bound. It is intriguing to further decide how lead ions are transported from the outside via the membrane and how they are transferred from the cytoplasm to vacuoles. As of now, results in this report are just morphological observations.

To corroborate the results of the above observation, the result of a Pb negative control need to be investigated in which the fungus grew in the same medium without the addition of Pb. No lead particles were observed on sections

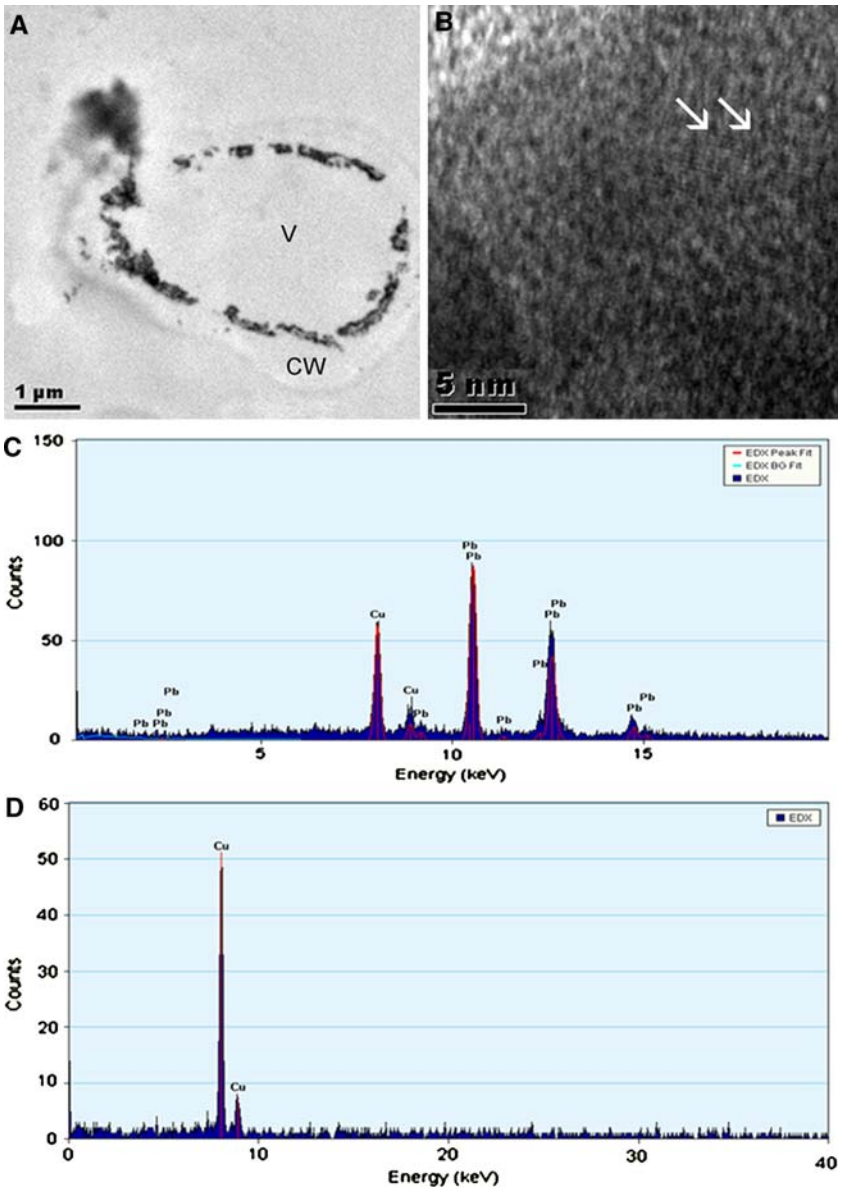

Fig. 3 EDX analysis of lead particles. **a** Section observed at 300 KV; the black ring is the result of intracellular lead accumulation. **b** The result of SAEF detection on the ring, showing the crystalline structure of the deposit; *arrow head* points to the lattice. **c** EDX result of the black deposit, showing the composition as lead. **d** EDX result of the center vacuole to show the background of copper derived from the copper grid

of the control (Fig. 2e). As a control for U staining, no additional particles of high electron density were produced by U staining to the sections (Fig. 2f).

To reconfirm that the particles of high electron density are the result of Pb accumulation, sections without U staining were observed using a higher power TEM (model: Tecnai F30) equipped with energy dispersive X-ray spectroscopy (EDX). Under 300 kV, the black particles were subjected to EDX analysis and selected area electron diffraction (SAED). EDX analysis confirmed that the black particle formed ring was derived from Pb, and no P element was detected (Fig. 3c); other detected elements, except Cu which is the background signal, were C, N and O, and the atomic ratio of C/N/O/Pb is 3.276/2.333/2.543/91.849. The percentages of C, N and O were very low and might result from the background. This is different than the result of *C. cladosporioides* which could accumulate manganese as a complex with phosphorus (Shao and Sun 2007). In addition, SAED discovered the crystalline lattice of lead particles (Fig. 3b). These results demonstrated that the particles in Figs. 1 and 2 contained a high percentage of crystalline lead, but whether it is in element form needs to be investigated further.

In the case of *C. lacera* (ATCC 34603), more than 90% of lead was trapped on the cell surface, while only 1.7–5.5% of the total Pb was found in the intracellular fraction (Taboski et al. 2005). Our results imply that both biosorption and bioaccumulation of lead contributed to the high tolerance of the fungus Psf-2. We cannot judge which mechanism is more important for metal trapping, but intracellular sequestration and compartmentalization seemed to be the main strategies of metal resistance. The absorption outside cell wall is probably the binding of lead ions to EPS on the cell surface (Bhaskar and Bhosle 2006). This is a quick, non energy consuming process, while intracellular bioaccumulation must be dependent on an active transport system (Bruins et al. 2000), first via the membrane to the cytoplasm and then from the cytoplasm to the vacuoles.

Psf-2 is the first fungus capable of accumulating a large amount of lead intracellularly. Results in this report together with the manganese-resistant *Cladosporium cladosporioides* which could sequestrate Mn intracellularly with phosphorus (Shao and Sun 2007) suggested that intracellular accumulation is an important mechanism for fungi from deep sea sediment to resist heavy metals. These fungi are a potential treatment for heavy metal pollution.

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