BioMetals (2007) 20:93–105
DOI 10.1007/s10534-006-9018-y

Stress response in two strains of the aquatic hyphomycete *Heliscus lugdunensis* after exposure to cadmium and copper ions

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Received 7 June 2005; accepted 19 May 2006

Key words: aquatic hyphomycetes, *Heliscus lugdunensis*, glutathione, cadmium, copper

Abstract

Biochemical responses to cadmium (Cd^{2+}) and copper (Cu^{2+}) exposure were compared in two strains of the aquatic hyphomycete (AQH) *Heliscus lugdunensis*. One strain (H4-2-4) had been isolated from a heavy metal polluted site, the other (H8-2-1) from a moderately polluted habitat. Conidia of the two strains differed in shape and size. Intracellular accumulation of Cd^{2+} and Cu^{2+} was lower in H4-2-4 than in H8-2-1. Both strains synthesized significantly more glutathione (GSH), cysteine (Cys) and γ-glutamylcysteine (γ-EC) in the presence of 25 and 50 μM Cd^{2+}, but quantities and rates of synthesis were different. In H4-2-4, exposure to 50 μM Cd^{2+} increased GSH levels to 262% of the control; in H8-2-1 it increased to 156%. Mycelia of the two strains were analysed for peroxidase, dehydroascorbate reductase, glutathione reductase and glucose-6-phosphate dehydrogenase. With Cd^{2+} exposure, peroxidase activity increased in both strains. Cu^{2+} stress increased dehydroascorbate reductase activity in H4-2-4 but not in H8-2-1. Dehydroascorbate reductase and glucose-6-phosphate dehydrogenase activities progressively declined in the presence of Cd^{2+}, indicating a correlation with Cd^{2+} accumulation in both strains. Cd^{2+} and Cu^{2+} exposure decreased glutathione reductase activity.

Abbreviations: GSH – reduced glutathione; GSSG – oxidized glutathione; γ-*EC* – γ-*glutamylcysteine;* POD – peroxidase; DHR – dehydroascorbate reductase; GR – glutathione reductase; G6PD – glucose-6-phosphate dehydrogenase

Introduction

High levels of heavy metals in the environment are associated primarily with anthropogenic activities. This type of pollution generally lowers biodiversity in terrestrial and aquatic ecosystems (Prasad 2001), but many bacteria and fungi have evolved the ability to live in such stressful habitats (Lovley 2000). To survive metal exposure in their natural environment, bacteria and fungi have developed a number of resistance mechanisms (Lovley 2000; Mills 2002). Fungi can sequester, mobilize, or transform various ions (Gadd 2001; Gadd & Sayer 2000) and thereby control the biogeochemical mobility and behaviour of metals in aquatic environments. These microbial features might be exploited for reducing the environmental impact of mine waste (Gadd 2001; Rawlings 2002). Potential

applications in bioremediation have renewed interest in studying the effects of metals on fungi.

We have begun to investigate this topic in extremely polluted sites in the Mansfeld district in Central Germany (Krauss *et al*. 2003a, 2005a). This area has a long history of copper shale mining and smelting. Metalliferous sludge was deposited in open basins and ponds on slag heaps and mining waste dumps. Leachates enter surface and ground water and result in extremely high concentrations of toxic metal ions and metalloids in aquatic habitats. Our studies have demonstrated for the first time that surprisingly diverse communities of aquatic hyphomycetes (AQH) exist in polluted surface (Krauss *et al*. 1998; Sridhar *et al*. 2000, 2001) and subterranean waters (Krauss *et al*. 2003b, 2005b) of the region. AQH initiate the decay of allochthonous organic material in food webs of aquatic ecosystems. This raises three areas of concern: (1) How do human activities affect diversity and ecological functions of fungal communities? (2) Which strategies have allowed fungi to adapt their morphology and metabolism to heavy metal stress? (3) Which approaches may be useful for evaluating the impact of heavy metals and other pollutants on aquatic fungi? In laboratory experiments, Cd^{2+}, Cu^{2+} and Zn^{2+} inhibited both growth and reproduction of several aquatic hyphomycete species (Abel & Bärlocher 1984; Miersch *et al*. 1997; Krauss *et al*. 1998, 2003a). However, many fungi have evolved some tolerance of heavy metals by synthesizing sulfur-rich peptides (phytochelatines) using glutathione as building block (Gadd & Sayer 2000; Avery 2001; Gadd 2001; Mendoza-Cózatl *et al*. 2005) and gene encoded, cysteine-rich proteins (metallothioneins) (Clemens *et al*. 2003; Cobine *et al*. 2004; Bellion *et al*. 2006). The question remains whether this is sufficient to maintain ecological functions at sites with chronically high pollution. Interestingly, extracellular fungal ligninolytic and cellulolytic enzymes are regulated by heavy metals at the level of transcription as well as during their activity (Baldrian 2003).

Oxidative stress in biological systems includes the formation of different reactive oxygen species (ROS) and generating processes (Sugiyama 1994; Stochs & Bagchi 1995; Avery 2001; Pócsi *et al*. 2004; Mittler *et al*. 2004). Because of its redox properties, Cu^{2+} can catalyse the formation of ROS. In contrast, metals without redox capacity, such as Cd^{2+}, are more likely to influence the cellular redox status by reducing the antioxidant glutathione (GSH) pool. Thiols are essential agents in cellular redox signalling and control in animals (Moran *et al*. 2001), plants (Noctor *et al*. 2002) and fungi (Pócsi *et al*. 2004). Some aquatic hyphomycetes respond to Cd^{2+}, but not to Cu^{2+} or Zn^{2+}, by increased synthesis of SH-containing compounds (Miersch *et al*. 1997, 2001, 2005).

The antioxidant defence system in several plants (Mittler *et al*. 2004) and fungi (Amicarelli *et al*. 1999; Avery, 2001; Pócsi *et al*. 2004) has been shown to consist of several enzymes and low molecular weight antioxidants. However, nothing is known about the enzymatic background of the antioxidative response of aquatic hyphomycetes to heavy metals. This was the objective of the current study: we investigated the effects of Cu^{2+} and Cd^{2+} exposure on the activity of selected antioxidant enzymes (peroxidase, GSH reductase, dehydroascorbate reductase and glucose-6-phosphate reductase) of two *Heliscus lugdunensis* strains. We tried to correlate these responses with fungal thiol content and the capacity to accumulate intracellular heavy metals.

Methods

Organisms and sites of their isolation

The strains were isolated from two sites in the Mansfelder Land area, Germany. *Heliscus lugdunensis* (Sacc. et Thérry) strain H4-2-4 was isolated from a highly polluted site ('Stadtborn Hergisdorf') coordinates H4: x-4463985, y-5712061; Transverse Mercator Bessel system (Krauss *et al*. 2001) and strain H8-2-1 was isolated from a moderately polluted site (Glume Brook near Lutherstadt Eisleben: coordinates H8: x-4467731, y-5711315). Leaves of alder (*Alnus glutinosa*) were picked up and collected in autum in the Botanical Garden of the Martin-Luther-University Halle-Wittenberg and air dried, and autoclaved leaf discs (diameter 1.5 cm) were placed in nylon mesh bags and exposed for 4 weeks in October 1997. After recovery from the stream they were placed in Petri dishes with distilled, sterile water and incubated at 10 °C. After 2–6 days, they were searched for conidiophores under a stereomicroscope. To isolate pure cultures, individual spores were

picked up with capillary pipets, and transferred aseptically to agar plates (Krauss *et al.* 1998, 2001) [1% (w/v) malt extract (MERCK); 1.5% (w/v) agar; 200,000 units Penicillin and 200 mg Streptomycin (Sigma) in 20 ml sterile 0.9% (w/v) NaCl solution per 500 ml water; pH: 5.6–5.8]. Mycelia of the fungal strains are periodically transferred to malt agar plates (1% malt extract, 1.5% (w/v) agar) without heavy metals. The two sites from which the fungal strains were isolated are characterized by different heavy metal contents (H4: cadmium ca. 25 μM; copper ca. 210 μM; manganese ca. 350 μM; zinc ca. 40,000 μM. H8: cadmium ca. 0.05 μM; copper ca. 0.3 μM; manganese ca. 7 μM; zinc ca. 15 μM); additional water–chemical characteristics are listed in (Krauss *et al.* 1998, 2001).

Liquid culture of fungi

To investigate the effect of heavy metals, the fungi were grown in liquid medium (0.5% (w/v) malt extract, Merck; 0.1% (w/v) peptone, Difco). An agar plug (∅ 7 mm, 1% (w/v) malt extract, Merck, 1.5% (w/v) agar), overgrown with fungal mycelium was homogenized in 1 ml distilled, sterilized water. One millilitre of the homogenate was used to inoculate 75 ml medium in a 200-ml Erlenmeyer flask. The strains were incubated at 10 °C on a rotary shaker (120 rpm) in the dark. Periodically, mycelia were harvested on a Whatman filter No. 3 (for sampling schedule, see Figure 2), washed for 10 min with distilled water, dried to constant mass and weighed.

Scanning electron microscopy of spores

To induce sporulation, three agar plugs, overgrown with mycelium, were transferred to 150 ml sterilized, distilled water and aerated for 2 days at 20 °C. The spores were collected on 3 μm polycarbonate filters (Poretics, Inc.) and frozen on a metal block cooled in liquid nitrogen slush. They were then freeze-dried overnight at ca. 0.001 Pa. Phosphorus pentoxide was included in the freeze drier chamber as an additional drying agent. For scanning electron microscopy (SEM) observation, spores on the filter were fixed to aluminium specimen stubs using double-sided tape. The edges were rimmed with colloidal graphite. Specimens were then coated with ca. 20 nm gold in a Hummer 6.2 sputtering unit (Anatech. Ltd.) with argon as the source gas. The specimens were examined using a JOEL JSM-5600 SEM at the Digital Microscopy Facility, Mount Allison University using an accelerating voltage of 10 kV and 8 mm working distance. For spore measurements, 640×480 pixel, 5 s scan images were saved as digital files for 20 randomly selected spores of each strain. Length and width were measured to the nearest pixel using Adobe Photoshop 6.0 (Adobe Systems, Inc.) and converted to actual values using a conversion factor of 40 pixels μm^{-1} = 0.025 μm pixel^{-1} calculated from images of a magnification calibration specimen (Ladd Research Industries #64015) collected under identical conditions.

Biosorption and accumulation of cadmium and copper

After an initial incubation of 3 and 4 days, Cu or Cd (to final concentrations of 25 μM $CuCl_2$ or 50 μM $CdCl_2$, respectively) was added. On day 7 (H4-2-4) or 9 (H8-2-1), the mycelia were harvested on a Whatman filter No. 3, washed for 10 min with distilled water, 3 times with 20 mM $NiCl_2$ solution to remove extracellular Cd^{2+} and Cu^{2+} and again with distilled water (Brown & Wells 1988). The mycelium was then removed from the filter and dried 24 h at 65 °C. Fifty milligram portions of the mycelium were digested with 4 ml 65% (v/v) HNO_3 and 2 ml 30% (v/v) H_2O_2 in a microwave (Step 1: 130 bar, 10 min, TAP 5 min; Step 2: 275 bars, 20 min, TAP 10 min, 180 °C; CEM MDS 2100).

Heavy metal concentrations in the $NiCl_2$ washings (for biosorption) and in the mycelium digests (for accumulation) were determined by an atomic absorption spectrometer (ATI Unicam).

Thiol measurement

To extract thiols, freshly harvested mycelium was rinsed twice with distilled water and tamped dry between filter paper. The mycelium was crushed in liquid N_2 and appr. 50 mg was homogenized in 1.5 ml 0.1 N HCl (Gallego *et al.* 1996). After centrifugation for 20 min at $13{,}800 \times g$ and 4 °C the supernatant was stored on ice.

The glutathione content of the extract was determined enzymatically in the presence of

NADPH and DTNB (5,5′-Dithiobis(2-nitrobenzoic acid). This results in the production of thionitrobenzoic acid which is measured photometrically at 410 nm (Anderson 1985).

Reverse phase HPLC analysis of low molecular weight thiols and disulphides was completed as previously described in (Kranner & Grill 1996). Fifty milligram of the N_2 crushed mycelium was extracted with 1 ml 0.1 N HCl and centrifuged 15 min at 13,800 × *g*.

To measure cysteine, γ-EC and GSH, a 120 μl aliquot of the extract was mixed with 180 μl mM 2-(*N*-cyclohexylaminio)ethane-sulphonic acid (CHES) buffer (pH 9.3) and 30 μl 30 mM DTT and incubated 1 h on ice to reduce disulphides.

To determine GSSG, a 200 μl aliquote of the extract was mixed with 300 μl CHES and treated with 50 μl 5 mM NEM. The reaction was carried out at room temperature for 10 min. Excess NEM was removed by extracting 3 times with equal volumes of toluene. Thirty microlitre of 3 mM DTT was then added to 300 μl aliquots of NEM-treated probe and incubated for 1 h on ice. These aliquots were labelled with 10 μl 30 mM monobromobimane for 15 min in the dark. The reaction was stopped by adding 250 μl of 5% (v/v) acetic acid.

The samples were separated with a Merck-Hitachi LaChrom-HPLC system applying a 250 × 4 mm Lichrospher 60 RP select B silicagel (5 μm). The injection sample was 80 μl. The eluent was: (A): 2% (v/v) methanol/98% (v/v) water/0.25% (v/v) acetic acid (pH 4.3) and (B): 90% (v/v) methanol/10% (v/v) water/0.25% (v/v) acetic acid (pH 3.9) using a flow rate of 0.9 ml min^{-1}. The following gradient was used: 0–15 min 97–93% (v/v) (A), 15–30 min 93–0% (v/v) (A), 30–35 min 0% (v/v) (A), 35–45 min 0–97% (v/v) (A). The samples were detected by fluorescence (excitation 480 nm, emission 520 nm).

Enzyme assays

The mycelium was homogenized in buffer (1:5 w/v) containing 100 mM Tris/HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2% insoluble PVP with an ultrasonic probe (Bandelin Sonoplus HD 70) at 0–4 °C for 4× 40 s (100%). After centrifugation at 13,800 × *g* for 40 min the supernatant was passed through a Schleicher & Schüll filter (No. 597, 2.7 cm, and used for enzyme measurements). Proteins in the extracts were estimated following the method of Lowry *et al.* (1951).

All enzymes were assayed at 25 °C with a Shimadzu UV-1601 spectrophotometer.

Peroxidase (1.11.1.7.)

The measurement of peroxidase activity was modified from Alla (1995). The assay mixture contained 50 mM NaH_2PO_4 pH 5.5, 0.008% (v/v) *o*-dianisidine and 0.0003% (v/v) H_2O_2. Absorbance was measured at 460 nm (extinction coefficient: 11.3 mM^{-1} cm^{-1})

Dehydroascorbate reductase (EC 1.8.5.1.)

DHA reductase activity was determined by the method of Hossain & Asada (1984). The assay solutions contained 100 mM KH_2PO_4/NaOH, pH 6.4, 8 mM GSH and 0.15 mM dehydroascorbate acid. The reaction was monitored by following the decrease of absorbance at 260 nm (extinction coefficient: 14 mM^{-1} cm^{-1}).

Glucose-6-phosphate dehydrogenase (EC 1.1.11.49.)

Activity was recorded at 340 nm by the method described by Bergmeyer (1970). The standard assay mixture contained 50 mM Triethanolamine/HCl pH 7.6, 0.4 mM $NADP^+ \times Na_2$, 6 mM $MgCl_2$ and 1 mM glucose-6-phosphate. The reaction was monitored by following the increase of absorbance (extinction coefficient: 6.22 mM^{-1} cm^{-1}).

Glutathione reductase (EC 1.6.4.2.)

GSSG reductase activity was measured as described previously (Esterbauer & Grill 1978). The assay mixture contained 100 mM Tris/HCl buffer pH 7.4, 30 mM EDTA, 0.1% BSA, 0.4 mM GSSG and 0.2 mM NADPH (in 1% $NaHCO_3$). The blank did not contain GSSG. The oxidation of NADPH was followed at 340 nm (extinction coefficient: 6.22 mM^{-1} cm^{-1}).

Statistics

For statistical calculations, the computer program SPSS 11 for the Macintosh computer was used. Most data were evaluated with a Two-way

ANOVA, with fungal strain (2 levels: H8-2-1, H4-2-4) and metal exposure (3 levels: 0, 25, 50 μM) as factors. Separate analyses were done for Cd^{2+} and Cu^{2+} exposure. Percentages were arcsine transformed before calculations (Zar 1996).

Results

The conidia of the two strains of *H. lugdunensis* differed significantly in length (H4-2-4: 22 ± 3 μm; H8-2-1, 26 ± 3 μm; *t*-test; $p = 0.002$) and width (H4-2-4: 4.2 ± 0.3, H8-2-1: 3.4 ± 0.2 μm, $p < 0.0001$; Figure 1).

In liquid culture, *H. lugdunensis* strains H4-2-4 and H8-2-1 reached a maximum biomass of 1.7 and 1.5 mg dry mass per ml, respectively. The lag phase lasted longer in strain H8-2-1 than in strain H4-2-4. Growth curves and sampling dates are shown in Figure 2.

The two strains reacted differently to the presence of Cu^{2+} but not to the presence of Cd^{2+} (Table 1). The dry mass production of strain H4-2-4 increased by 40% (25 μM Cu^{2+}) and 30% (50 μM), respectively. No significant effect of Cu^{2+} exposure on biomass production was found for H8-2-1. When exposed to Cd^{2+}, biomass production of both fungal strains was significantly reduced.

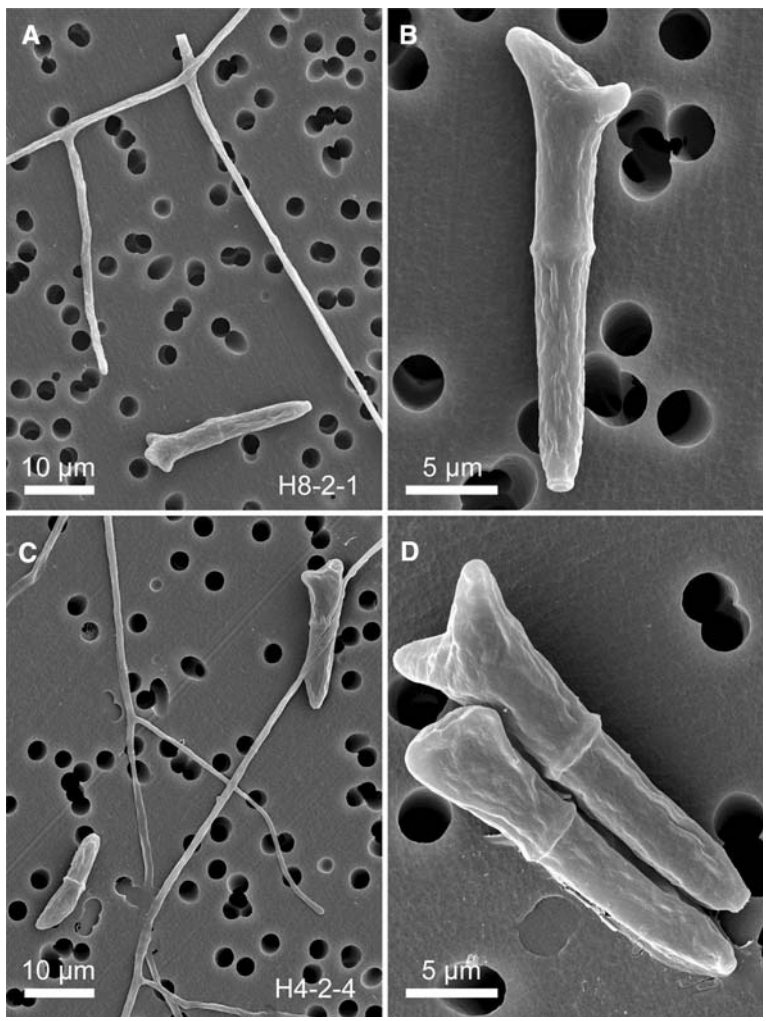

Figure 1. SEM images of representative conidia of *H. lugdunensis* strains H8-2-1 (A–B) and H4-2-4 (C–D).

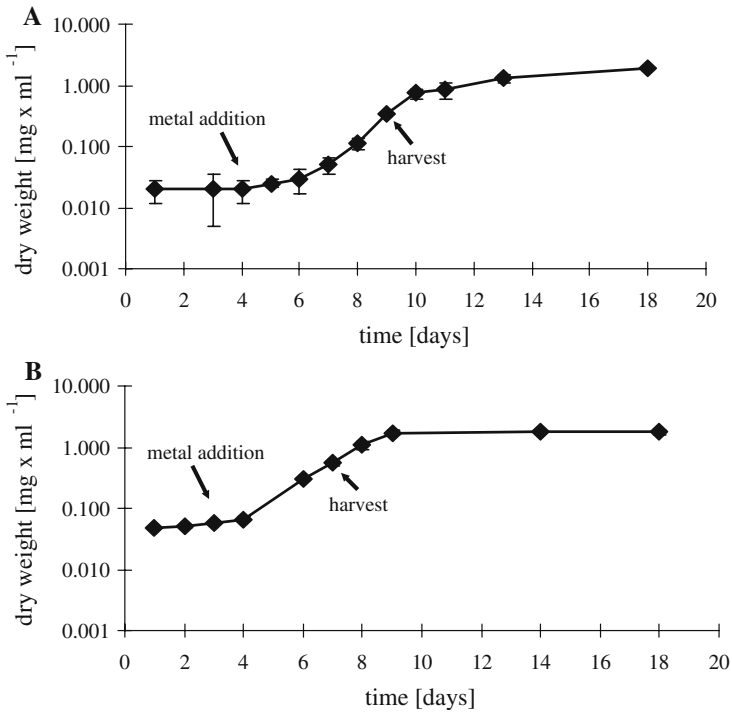

Figure 2. Growth curves of *H. lugdunensis* strains H8-2-1 (A) and H4-2-4 (B) in malt–peptone medium ($n = 3$; SD: H8-2-1 $\leq 20\%$; H4-2-4 $\leq 5\%$). Arrows bracket period used to study effects of heavy metal addition.

Both strains exposed to Cd^{2+} and Cu^{2+} accumulated significant, strain-specific amounts of heavy metal externally through biosorption and internally through intracellular accumulation (Figure 3). Mycelial biosorption increased with external Cd^{2+} and Cu^{2+} concentrations, but did not differ significantly between the two strains. No isotherms were calculated because the biomass was growing during the experimental period. Accumulation of Cd^{2+} and Cu^{2+} was significantly lower in H4-2-4 than in H8-2-1 (Figure 3).

Cd^{2+} exposure resulted in strain-specific increases in various thiol compounds, including cysteine, γ-glutamylcysteine and reduced GSH (Table 2). Oxidized glutathione, by constrast, was not significantly influenced by Cd levels. Thiol

Table 1. Biomass production by *H. lugdunensis* strains H8-2-1 and H4-2-4 in malt–peptone medium exposed to 3 levels of Cd^{2+} and Cu^{2+} ($n = 3$, ±SD). Significant effects were found for Cd concentrations and for strains in the Cu treatment ($p \leq 0.001$)

Metal	Concentration	Strain	Biomass
Cd^{2+}	0	H4-2-4	1.12 ± 0.18
	25		0.70 ± 0.12
	50		0.50 ± 0.09
	0	H8-2-1	0.81 ± 0.16
	25		0.46 ± 0.06
	50		0.32 ± 0.05
Cu^{2+}	0	H4-2-4	1.28 ± 0.15
	25		1.76 ± 0.13
	50		1.65 ± 0.17
	0	H8-2-1	0.79 ± 0.20
	25		0.72 ± 0.13
	50		0.69 ± 0.16

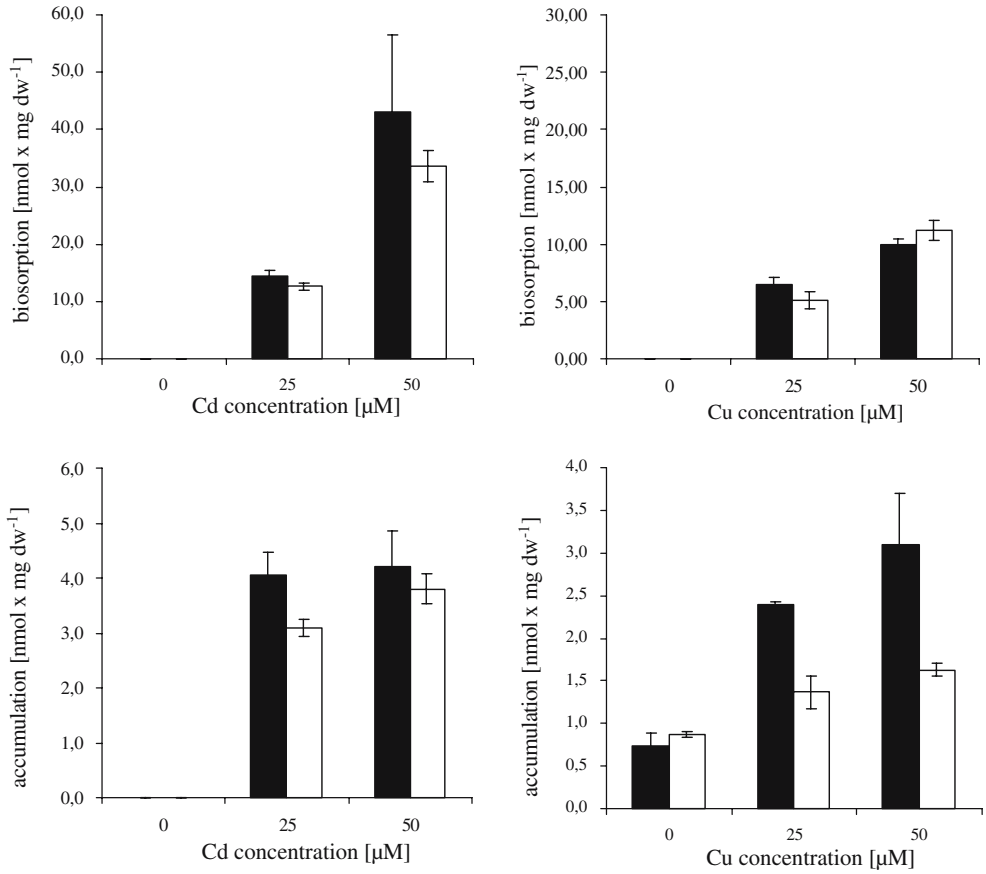

Figure 3. Cd^{2+} and Cu^{2+} biosorption and accumulation in *H. lugdunensis* H8-2-1 (■) and H4-2-4 (□). With Cd, accumulation varied with strain and concentration, and biosorption with concentration ($p \leq 0.048$); with Cu, both parameters were affected by strain and concentration ($p \leq 0.001$).

compounds increased more in the strain isolated from the highly contaminated site. Levels of reduced glutathione did not change under Cu^{2+} exposure (data not shown).

Heavy metal stress affected the redox status of cells, which can be expressed as the ratio of GSH:GSSG. As shown in Table 3, this ratio increased parallel to Cd^{2+} concentration from

Table 2. Effect of cadmium on levels of cysteine (Cys), reduced (GSH) and oxidized (GSSG) glutathione and γ-glutamylcysteine (γ-EC) in *H. lugdunensis* strains H8-2-1 and H4-2-4 ($n = 4$; ±SD). All four variables differed significantly among the two strains; all but GSSG were significantly affected by metal concentration ($p \leq 0.001$)

		Cd^{2+}		
		0	25	50
H8-2-1	GSH	5.70 ± 0.58	8.13 ± 0.55	8.88 ± 1.68
	GSSG	0.11 ± 0.01	0.13 ± 0.02	0.15 ± 0.02
	Cys	0.20 ± 0.02	0.32 ± 0.06	0.45 ± 0.05
	γ-EC	0.08 ± 0.01	0.10 ± 0.03	0.21 ± 0.06
H4-2-4	GSH	5.56 ± 1.64	12.53 ± 1.59	14.59 ± 2.01
	GSSG	0.18 ± 0.05	0.21 ± 0.05	0.23 ± 0.03
	Cys	0.22 ± 0.06	0.97 ± 0.30	1.41 ± 0.44
	γ-EC	0.12 ± 0.05	0.45 ± 0.15	0.67 ± 0.23

32 to 64 in H4-2-4 (50 μM) and from 54 to 64 (25 μM) in H8-2-1. Strain and concentration, as well as their interaction, significantly affected this ratio.

The ratio of intracellular antioxidants is regulated by various enzymes scavenging reactive oxygen species. Peroxidase (POD), dehydroascorbate reductase (DHR), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) are all involved in the glutathione-redox cycle representing an important regulatory part of oxidative stress (Noctor & Foyer 1998). Under the influence of Cd^{2+} and Cu^{2+}, the activities of most of these enzymes changed significantly.

Discussion

Heavy metal pollution typically impoverishes fungal communities in terrestrial and aquatic habitats (Gadd 1993; Krauss *et al*. 1998, 2001, 2003a, b, 2005a; Sridhar *et al*. 2000, 2001). Over time, local populations may diverge genetically in response to natural selection specific to their habitat. The ability of some fungi to survive in high concentrations of essential or non-essential toxic metal ions hinges on an array of tolerance/resistance mechanisms that are not fully understood. The aim of this work was to compare two strains of the aquatic hyphomycete *Heliscus lugdunensis* (Sacc. et Thérry) isolated from two sites in the Mansfelder Land (Central Germany) differing in their degree of heavy metal contamination (Krauss *et al*. 1998, 2001, 2003a). We were interested in their reaction toward two common pollutants, cadmium and copper. Cd^{2+} is a trace pollutant toxic for all organisms. Cu^{2+} is an essential micronutrient, however, at high concentrations, Cu^{2+} ions interfere with numerous biochemical processes. Both metals can modify oxygen metabolism in fungi (Avery 2001) and plants (Shaw *et al*. 2004) by inducing the formation of free radicals and reactive oxygen species. Like other organisms, fungi possess a number of antioxidant molecules and enzymes as protection against oxidative stress. Nevertheless, elevated concentrations of heavy metals may reduce growth of fungi and damage fungal cells. At extracellular concentrations of 25 and 50 μM Cd^{2+}, no significant growth inhibition was measured in the two *H. lugdunensis* strains. On the other hand, 25 μM Cu^{2+} enhanced growth of H4-2-4, but not of H8-2-1 (Table 1). Biomass accumulation reflects complex biochemical adaptations beginning with biosorption and accumulation. We also observed some morphological differences. When exposed to Cd^{2+}, swollen cells were observed in both strains (light microscope, data not shown), similar to reports from *Fusarium* sp. and *Alternaria tenuis* (Gharieb 2001) or *Mucor rouxii* for Cu^{2+} (Gardea-Torresdey *et al*. 1997). The function of these Cd^{2+} induced structures is unknown. Possibly, Cd^{2+} binds to functional groups in the cell wall that are involved in cell proliferation. This response, however, does not appear to be specific; stress due to low pH, potassium or oxygen deficiency, or high osmotic pressure can result in similar changes in fungal cell morphology (Gharieb 2001). Mycelial morphology was not affected by exposure to Cu^{2+}.

Fungal cells have evolved sophisticated mechanisms to buffer toxic concentrations of heavy metals in their environment and to regulate intracellular concentrations. To gain insight into these processes, we measured biosorption and accumulation of Cd^{2+} and Cu^{2+} in the two strains. In contrast to *M. rouxii* (Gardea-Torresdey *et al*. 1997), we measured no correlation between metal tolerance and biosorption rate (though there was a significant interaction between strain and metal concentration). However, the strain-specific accumulation rates of the heavy metals in H4-2-4 (Figure 3) reflect differences in effective metal exclusion or removal mechanisms. Fungi take up heavy metals by transport systems of varying specificity (Gadd & Sayer 2000; Gadd 2001; Van Ho 2002). *Saccharomyces cerevisiae* has been used as a model organism to study the processes of metal homeostasis and resistance (Van Ho 2002). This yeast has a low and a high (CTR1 and CTR3) copper affinity transport system (Van Ho 2002). Gomes *et al*. (2002) showed that the transport of Cd^{2+} in *S. cerevisiae* is carried out through the zinc transporter ZRT1. These transport systems could be regulated in *H. lugdunensis* strains in such manner that only a small amount of heavy metals is taken up. Different transport systems may be involved in cadmium and copper uptake of the two strains isolated from differently contaminated sites.

Increased biosorption, reduced metal uptake and changes in intracellular trafficking and sequestration of toxic metals are the most common

mechanisms leading to metal resistance in fungi (Gadd & Sayer 2000; Gadd 2001; Van Ho *et al.* 2002). Some antioxidants, such as GSH, thiols, carotinoids and ascorbate may play a role in protection against heavy metals by preventing attack on labile macromolecules by free radicals that are formed during various metabolic reactions leading to oxidative stress (Avery 2001; Shaw *et al.* 2004). Many investigators have shown that GSH plays an important role in the response of yeasts to oxidative stress (Penninckx 2002). In the present study, the level of the antioxidant GSH was strain-specific. At a Cd^{2+} level of 50 μM, GSH level increased by 262% in H4-2-4 and by 156% in H8-2-1 (Table 2). This is similar to the findings of Pawlik-Skowronska (2003) who found that the Zn^{2+} tolerant alga *Stigeoclonium tenue* from a highly contaminated site produced more thiols than the same species from an unpolluted site. The differences between the two *H. lugdunensis* strains also confirm results from other aquatic hyphomycetes (Miersch *et al.* 1997, 2001, 2005). Another *H. lugdunensis* strain, harvested during the stationary phase, increased its internal thiol pool by 4 μmol g^{-1} dry mass at Cd^{2+} concentrations of up to 200 μM Cd^{2+} (Miersch *et al.* 1997), while exposure to copper had no effect. *Varicosporium elodeae* and *Tetracladium marchalianum* behaved in a similar manner (Miersch *et al.* 1997, 2005). In the zygomycete *Mucor racemosus*, cadmium caused an increase of the thiol pool with a simultaneous decrease of glutathione and induction of phytochelatins whereas copper had no effect (Miersch *et al.* 2001). In *M. racemosus*, extended Cd^{2+} exposure (10 days, 100 μM), resulted in a GSH:GSSG ratio (representing the cellular redox state) of 53 compared to a control value of 33 (Miersch *et al.* 2001). In the current study, this ratio increased from 32 in the control to 64 under Cd^{2+} exposure (50 μM) in H4-2-4, and from 54 to 58 in H8-2-1 (Table 3). Under oxidative stress, glutathione may shift to its oxidized form due to increased GSH oxidation and/or decreased GSSG reduction, catalysed by glutathione reductase (Devi & Prasad 1998; Noctor & Foyer 1998). The increased GSH:GSSG ratio (Table 3) suggests an increase of GSH level without dramatic changes in GSSG levels.

We hypothesize that under Cd^{2+} stress a substantial proportion of the reduced glutathione was removed from the glutathione pool to detoxify the metal by intracellular chelation. For both structural and reactivity reasons it seems reasonable to assume that GSH is able to chelate heavy metals. Exposure to 100 as well as 200 μM Cd approximately doupled the respective GSH/GSSG ratio in the white rot basidiomycete *Abortiporus biennis* which could indicate a role of GSH in initial Cd chelation (Jarosz-Wilkołazka *et al.* 2005). In *S. cerevisiae*, Cd^{2+} detoxification depends primarily on binding by glutathione as bis(glutathionato)Cd and the sequestration of these complexes into the vacuole mediated by the ABC-transporter YCF1 (Li *et al.* 1997). Some evidence for intracellular metal chelation by GSH was given for Cd stressed ectomycorrhizal fungus *Paxillus involutus* by (a) the remarkable increase of glutathione and its precursor γ-glutamylcysteine, accompanied with the complete lack of PCs (Ott *et al.* 2002; Courbot *et al.* 2004; Bellion *et al.* 2006), and (b) the high Cd content in vacuoles (Blaudez *et al.* 2000; Ott *et al.* 2002), possibly mediated by a bis(glutathionato)Cd transporter. In bryophytes, no phytochelatins occur under Cd^{2+} exposure, but there is evidence for cytosolic localization of GSH–Cd complexes (Bruns *et al.* 2001). In *Schizosaccharomyces pombe*, metal binding by phytochelatins is the main cadmium detoxification mechanism (Clemens & Simm 2003). For Cd^{2+}, two types of peptide complexes exist in *S. pombe*. A low molecular weight complex consists of phytochelatin–Cd complexes, while a higher molecular weight complex also contains acid soluble sulfide (Ow *et al.* 1994). In *Candida glabrata*, glutathione and phytochelatins capped CdS crystallites occur (Dameron *et al.* 1989). Under the cultivation conditions described no PCs could be detected in *H. lugdunensis*.

Increased GSH content in both *H. lugdunensis* strains exposed to Cd^{2+} parallels the reactions in other aquatic hyphomycetes (Miersch *et al.* 1997, 2001, 2005). To counteract the toxicity of reactive oxygen species generated by excessive Cd^{2+} and Cu^{2+} exposure, a highly efficient antioxidative defense system is present in cells of all organisms, *e.g.*, in humans (Sugiyama 1994; Moran *et al.* 2001), plants (Mittler *et al.* 2004) and microorganisms (Cherry 2000; Avery 2001). It is characterized by consistent responses by a number of enzymes. To begin with, the uptake of toxic quantities of heavy metals induces peroxidases. In the current study, the highest increase of POD

activity in both strains was observed during 25 μM Cd^{2+} exposure (Figure 4). However, enzyme activity was lower at 50 μM Cd^{2+}, which suggests that at higher intracellular Cd^{2+} concentrations (Figure 3), POD may be insufficient to protect the fungus against oxidative stress, and that other defence mechanisms may have to be to activated. Exposure to 25 μM Cu^{2+} did not change the POD activity in the two strains (Figure 4). Despite higher intracellular metal concentration at 50 μM Cu^{2+}, POD activity did not increase in H8-2-1, probably showing the effectiveness of POD. The decline of POD activity in H4-2-4 when comparing 50 and 25 μM Cu^{2+}, is accompanied by an unchanged intracellular Cu^{2+} concentration. This may indicate successful adaptation of this particular strain. POD activity could be an early indication of Cu^{2+} toxicity in *H. lugdunensis* strains.

The presence of the ascorbate–glutathione cycle in almost all cellular compartments tested to date in plants (Mittler *et al.* 2004; Noctor & Foyer 1998), microorganisms (Pócsi *et al.* 2004) and yeasts (Avery 2001) suggest that it plays a vital role in controlling the level of reactive oxygen intermediates. Maintaining a high ratio of reduced to oxidized states of ascorbic acid and glutathione is important for cellular metabolism. Dehydroascorbate (DHA) is reduced to ascorbate by DHA reductase, with GSH being used as reducing substrate. Increased DHA reductase activity could only be measured in H4-2-4 at 25 μM Cu^{2+} (Figure 4). Obviously, under Cu^{2+} exposure more DHA is needed to participate in the cyclic transfer of reducing equivalents. Both strains showed a decrease of DHA reductase activity during Cd^{2+} exposure. The reason is unclear. Possibly, high intracellular toxic metal ion concentrations inhibit the enzyme.

Table 3. GSH:GSSG ratio in *H. lugdunensis* strains H8-2-1 and H4-2-4 in relation to external Cd^{2+} concentration. Both fungal strain and metal concentration had a significant effect ($p \leq 0.01$)

Cd concentration (μM)	GSH:GSSG	
	H8-2-1	H4-2-4
0	54	32
25	64	61
50	58	64

Glutathione reductase (GR) is a key enzyme of the ascorbate–glutathione cycle and regulates the intracellular homeostasis of glutathione. GR plays an important role in regulating the GSH:GSSG ratio. Its activity depends strictly on the cellular GSH level (Carlberg & Mannervik 1985). In plant cells (*Hordeum vulgare*), Finkemeier *et al.* (2003) observed a 50% reduction of glutathione reductase activity depending on external N availability. Vitoria *et al.* (2001) showed increased enzyme activity in radish, but the intracellular Cd^{2+} concentration were only approx. 2% of the values in this study. In the ectomycorrhizal basidiomycete *Paxillus involutus*, GR activity depended on Cd^{2+} concentration and exposure time (Ott *et al.* 2002). Decreased GR activity should increase GSSG levels and lower the GSH:GSSG ratio. This was not the case in both strains investigated (Table 3).

Another reason for decreased glutathione reductase activity could be a lack of redox equivalents such as NADPH. One enzyme producing redox equivalents is the glucose-6-phosphate dehydrogenase, which catalyses the first reaction in the pentose phosphate way. Izawa *et al.* (1998) describes these interactions between glutathione reductase, dehydroascorbate reductase, glucose-6-phophate dehydrogenase and NADPH for *S. cerevisiae* as a response to H_2O_2, causing oxidative stress. In the current study, concentrations of up to 50 μM Cu^{2+} and Cd^{2+} significantly depressed the activity of glucose-6-phosphate dehydrogenase (Figure 4). This could be due to a lack of redox equivalents, or, conversely, inhibition by high levels of redox equivalents. Direct interference by the metals can be excluded, since adding Cd^{2+} or Cu^{2+} to the test tube did not affect enzyme activities (data not shown).

Our findings add new information on the metabolism of aquatic hyphomycetes under heavy metal stress. The results indicate that Cd^{2+} and Cu^{2+} can disturb the oxidative balance in *H. lugdunensis*. The response varied with the fungal strain used and with the metal applied. Modulations of the antioxidant status may therefore be an important adaptive response to heavy metals in aquatic hyphomycetes. Together with different conidial morphologies, biochemical and physiological divergence suggests that the investigated strains may represent ecotypes that have evolved distinct genetic and physiological adaptations to differently contaminated habitats. More detailed

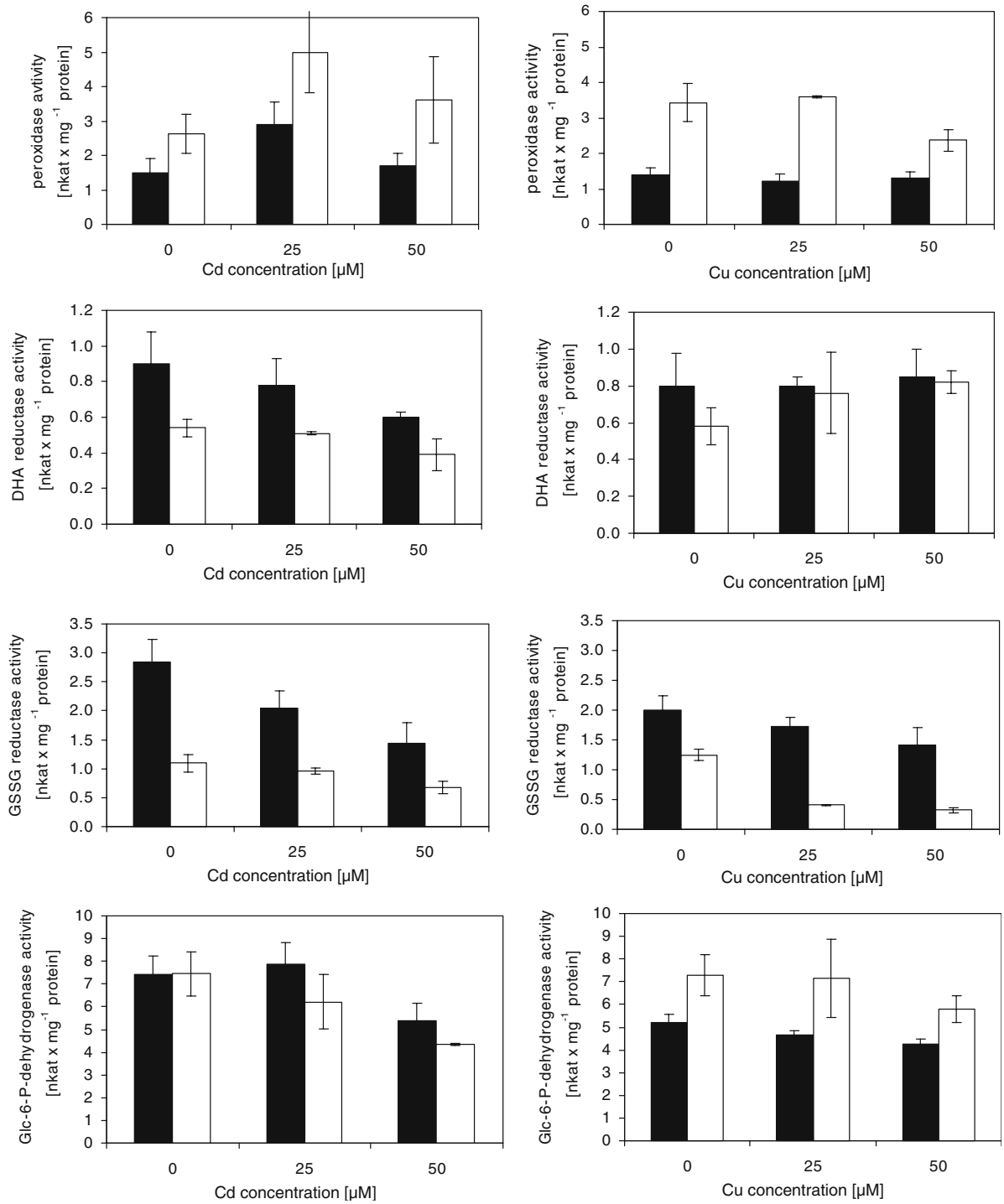

Figure 4. Enzyme activities under Cd^{2+} and Cu^{2+} exposure in *H. lugdunensis* strains H8-2-1 and H4-2-4 in the logarithmic phase (■ = H8-2-1, □ = H4-2-4; $n = 4$). With one exception (G6PD and Cd), all parameters were significantly affected by fungal strain ($p \leq 0.03$). The effect of concentration was significant for the following combinations: Cd and POD and DHA; Cu and GR and G6PD ($p \leq 0.046$).

analyses, involving a larger number of isolates from a range of habitats and addressing metal ion transport, intracellular trafficking and sequestration, and their genetic basis will be needed to allow a deeper understanding of the connection between heavy metal pollution and fungal responses.

Acknowledgements

Financial support by the Canadian-German Scientific and Technological Research cooperation (BMBF/WTZ No. ENV 99/1) and by NSERC (Canada) is gratefully acknowledged. We thank B. Krause (UFZ) and W. Püschel (Martin-Luther-University) for their excellent technical assistance.

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