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Cadmium and heat response of the fungus *Heliscus lugdunensis* isolated from highly polluted and unpolluted areas

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Summary. Induction of heat shock protein (Hsp) 70 and distinct metallothionein-like proteins (MTLPs) in response to Cd and heat treatment were studied in two strains of the aquatic hyphomycete *Heliscus lugdunensis*: Hl-H4, isolated from a heavy metal polluted site, and Hl-BB taken from an unpolluted area. Upon Cd-exposure, Hsp70 was actively synthesized in the strain Hl-H4, and to a much lower degree in the strain Hl-BB. The Hsp70-expression was time- and dose-dependent, reaching a maximum after 24 h incubation with 80 μM Cd. Upon heat-stress, a similar response was observed: a strong Hsp70-expression in Hl-H4, and only a marginal one in Hl-BB. The strains reacted to Cd-exposure by a specific, environmentally related induction of MTLPs, as shown by the highly sensitive bimane derivatisation method of SH-rich proteins. In Hl-H4, a strong expression of 11 kDa MTLP was registered, which followed strictly the induction pattern of Hsp70. This suggests interdependence of the induction mechanisms and roles of these stress proteins in metal resistance. On the contrary, in Hl-BB a weak expression of MTLP of about 20 kDa was observed, exhibiting completely different induction pattern. The results suggest that the specific induction of Hsp70 and/or distinct MTLPs in the range of 11 kDa in *H. lugdunensis* strain Hl-H4 are essential adaptive mechanisms to continuous heavy metal exposure.

Keywords: Cadmium – Heat stress – Metallothioneins – Aquatic hyphomycetes – *Heliscus lugdunensis* strains

Abbreviations: AA, acrylamide; AP, alkaline phosphatase; APS, ammonium peroxydisulphate; Bis-N,N′-methylene-bis-acrylamide; BCIP, 5-bromo-4-chloro-e-indolyl phosphate disodium salt; DTE, dithiothreitol; DTPA, bis-(2-aminoethyl)-amino-N,N,N′,N″,N″-pentaacetic acid; ECL, electrogenerated chemoluminescence; EDTA, ethylenediamine tetraacetic acid; NC, nitrocellulose; PMSF, phenylmethylsulphonylfluoride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; PBS, phosphate buffered saline; Hsp, heat shock protein; MT, metallothionein; MTLP, metallothionein-like protein; NBT, p-nitro blue tetrazolium chloride; TCA, trichloroacetic acid; TCEP, tris-(2-carboxyethyl) phosphine chloride; Tris, tris(hydroxymethyl)aminomethane

Introduction

In recent years, there has been a renewed interest in the role of fungi as potential bioremediators of heavy metal polluted environments (Gadd, 2001; Fomina et al., 2005). Fungi are capable of removing metals either by biosorption or bioaccumulation in the cytoplasm, binding them, e.g., by cysteine-rich peptides such as glutathione (GSH), metalthioneins (MTs) or phytochelatins (PCs) (Cobett and Goldsbrough, 2002; Jarosz-Wilkolazka et al., 2005). Special attention in this respect has been given to some aquatic fungi found in highly contaminated waters. The aquatic hyphomycete *Heliscus lugdunensis* was one of the most abundant species inhabiting heavy metal polluted sites (Krauss et al., 2005a, b; Sridhar et al., 2005). Heavy metal tolerance in filamentous fungi and their mechanisms of adaptation to polluted aquatic ecosystems are not well understood. In the filamentous fungus *Curvularia* sp. tolerance against Cd is acquired by biosorption on the mycelial surface (Vepachedu et al., 1997).

H. lugdunensis strains have been shown to respond to increased external Cd-concentrations by increasing the internal GSH-content, which is accompanied by rising levels of cysteine and γ-glutamylcysteine (Braha et al., 2007), as well as by the synthesis of some MTs and PCs (Jaeckel et al., 2005a, b). Yet, the detailed role of distinct MTs in the metal detoxification in fungi remains to be elucidated. Little is known about the participation of other stress proteins (e.g., heat shock proteins, Hsps) in these defense mechanisms. Hsps are ubiquitous tools of all living organisms to cope with stress-induced denaturation of other proteins (Wang et al., 2004).

In this work, we compared the Cd-stress and heat response of *Heliscus lugdunensis* strain H4-2-4, isolated from a highly polluted area with that of Hl-BB isolated from an unpolluted site. We focused our attention on

involvement of Hsp70 and distinct MTs in fungal defence and adaptation against heavy metal toxicity.

Materials and methods

Chemicals and Hsp70 antibody

Acrylamide; N,N′-methylene-bis-acrylamide-Solution (Rotiphorese gel 30, Roth, Karlsruhe); sodium dodecyl sulphate (Roth); Tricine (Roth); Tris (Roth); Temed- (N,N,N′,N′-tetramethylethylenediamine, Serva, Heidelberg), ammonium peroxydisulphate (Merck, Darmstadt); NBT, p-nitro blue tetrazolium chloride (USB Corp., Cleveland Ohio); BCIP, 5-bromo-4-chloro-e-indolyl phosphate (USB Corp., Cleveland Ohio); DTPA, bis-(2-aminoethyl)-amino-N,N,N′,N″,N″-pentaacetic acid (Sigma-Aldrich, München); monobromobimane (Fluka, Darmstadt); polyclonal antibody against cytosolic Hsp70 of tomato (*Lycopersicon peruvianum*) was a gift of Neumann et al. (1987); rabbit anti-Hsp70 alkaline phosphatase conjugate (Biomol, Hamburg); Western-Light chemoluminescence-test (CDP-Star, Tropix, Invitrogen, Carlbad CA, Cat-No. SO-WOO8).

Fungal strains and cultivation

Strains of *Heliscus lugdunensis* Sacc. et Therry were isolated from single conidia from following sites: strain Hl-BB, from a non-polluted stream (Boss Brook near Amherst, Nova Scotia, by Prof. F. Bärlocher, Mount-Allison-University Sackville, Canada (Bärlocher, 1987)); strain H4-2-4, from a polluted location (Hergisdorf near Eisleben, Saxony-Anhalt, Germany, by Krauss et al. (1998, 2001)). The strains are available from DSMZ Braunschweig (HL-BB: DSM 18483) or Dr. G. Krauss (H4-2-4, Department Groundwater Microbiology, UFZ, Centre for Environmental Research, Leipzig-Halle, Germany), abbreviated to Hl-H4.

All cultures were maintained on malt extract agar (0.5% malt extract, 0.1% peptone, 1.5% agar). To examine the effects of heavy metals on biomass production, the fungi were grown in broth cultures (0.5% malt extract, 0.1% peptone; 75 ml medium in 200 ml Erlenmeyer flasks). The cultures were inoculated with homogenized agar slices overgrown with a 14 days old culture; the total fungal biomass introduced per flask was 1–1.3 mg. The flasks were incubated on an orbital shaker ($14 \pm 0.7\,^{\circ}C$; 120 rpm). Cd^{2+} was added as chloride salts (Sigma) in the first variant, at beginning of cultivation, and in the second variant, at 4th day of cultivation. Standard solutions were filter sterilized (0.2 µm membrane filters) before adding to achieve final metal concentrations of 40, 80 and 120 µM in the cultures. The distinct procedure of time and temperature arrangements in induction experiments of Hsp70 and thiol proteins (metallothioneins or metallothionein-like proteins) are given in the corresponding legends of figures.

Determination of dry mass

To determine growth, mycelia incubated for 12 d in three parallels without or with Cd at a final concentration of 80 µM were used (log phase) and then harvested by filtration (Merck, filter 0869) after 2, 4, 6, 8, 10 and 12 days. Mycelia were rinsed twice with distilled water, blotted dry between filter papers, weighed (fresh weight) and used for analysis. To determine wet to dry weight ratio, pre-weighed fresh mycelia were exposed to 80 °C for at least 2 h, and weighed again. The standard deviation of the conversion factor was 15–18%. The influence of Cd on growth of the *Heliscus* strains Hl-BB and Hl-H4 is shown in Fig. 2 by average values and standard deviations.

Protein determination

Hundred microliters of the supernatant were mixed with 1 ml of 5% TCA and left overnight at 4 °C to precipitate the proteins. The precipitate was washed 1-time with 1% TCA and 3-times with water, and the amount of protein determined according to Lowry et al. (1951).

Isolation of cytosolic protein to identify Hsp70

The mycelia were harvested by filtration and the wet mass was homogenized under liquid N_2 by stirring using mortar and pestle. The material was extracted with 50 mM Tris–HCl (pH 8) containing 3 mM EDTA, 10 mM DTE and 0.2% PMSF. The homogenate was centrifuged at 8500 *g* for 10 min and aliquots were used for protein determination and sample preparation.

Sample preparation to identify Hsp70

The proteins were precipitated by a pre-chilled acetone at −20 °C overnight, centrifuged at 5000 *g* for 30 min and dissolved as follows: For SDS-PAGE, 100 µg of protein were dissolved in sample-buffer (50 mM Tris–HCl, pH 6.8, 10% (w/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol) to obtain a final concentration of 2 µg/µl. Before electrophoresis, to the samples 2 µl of 50% bromphenol blue were added.

Sample preparation to identify sulfur-rich proteins (metallothioneins)

The mycelia were harvested and homogenized as above. The frozen material (15–30 mg fresh mass) was filled in pre-chilled (−20 °C) septum-vials, 100 µl of 500 µM TCEP were added and the proteins were reduced for 1 h at room temperature in darkness. Then, to each sample 0.4 ml of 5.8 mM monobromobimane in 50 mM Hepes-NAOH-buffer at pH 8, containing 5 mM DTPA and 50% acetonitrile, was added. The derivatization and sample preparation for SDS-PAGE followed the procedure of Fan et al. (2004).

For SDS-PAGE, 100 µg of protein were dissolved in sample-buffer (50 mM Tris–HCl, pH 6.8, 10% (w/v) glycerol, 4% (w/v) SDS) to obtain a final concentration of 2 µg/ µl. Before electrophoresis, to the samples 2 µl of 50% bromphenol blue were added.

SDS-PAGE

For Hsp70 analysis, one-dimensional SDS-polyacrylamide gel electrophoresis was carried out in 5% (w/v) stacking gel and 12.5% (w/v) separating gel, or in a 12–17.5% gradient gel, according to Laemmli (1970). The proteins were stained with Coomassie Brilliant Blue R-250 (Serva, Heidelberg) according to Weber and Osborne (1969) or silver, according to Blum et al. (1987). To analyze sulfur-rich-proteins (MT), the proteins were separated by one-dimensional PAGE on a modified Tricine-urea gel, according to Schägger and Jagow (1987): The stacking gel contained 4%AA; 0.44% Bis; 0.31 M Tris–HCL, pH 8.5; 0.4% SDS, the separation gel 12–20% AA, 1.9% Bis, 4.6 M urea, 0.76 M Tricine–Tris, pH 8.5; 0.1% SDS. To 7.5 ml separation gel mixture 2 µl Temed and 20 µl 10% APS were added and the mixture left to polymerize overnight at 8 °C. For relative molecular weight calibration, recombinant proteins prestained (Fermentas SMO671, 11, 17, 24, 30, 40, 55, 70, 100, 130, 170 kDa or SM1811, 10, 15, 27, 35, 55, 70, 100, 130, 250 kDa) were used.

The gels were run in a Minigel-tool (Biometra, Göttingen) for about 1 h at 80 V, 15–18 mA and about 1.5–2 h at 100 V, 28–50 mA. After finishing of separation, the gels were stained using silver-reagent or coomassie as indicated above, and prepared for Hsp70 analysis by Western-blotting. In the case of monobromobimane-conjugated proteins, the gels were analyzed by UV-light (UV-Gel-Yet-Imager, INTAS) and quantified by 1-D-Image-System (Biotec-Fischer).

Western blot

Proteins were transblotted from gels onto nitrocellulose membranes (0.2 or 0.45 µm, Schleicher and Schüll, Germany) for 2 h at 300 mA, and 1 h at

350 mA using 0.02 M NaH_2PO_4, pH 6.4 in a tank-blot tool. The immunoblotting procedure was carried out as described by Neumann et al. (1987) using a polyclonal anti-rabbit Hsp70-antibody. After blotting, the proteins on the membrane were stained by Ponceau S, washed thrice with water, once with PBS-solution and blocked for 1 h at 37 °C with 1% non-fat-milk (Bio-Rad, Germany) in PBS-solution, pH 7.5 and 0.1% Tween 20. Thereafter, the membrane was washed for 1 h with PBS and probed for 1 h with a polyclonal antibody against cytosolic Hsp70 (*Lycopersicon peruvianum*; 1:1000) as indicated. After washing again with PBS for 1 h, the membrane was incubated for 1 h with AP-conjugated secondary goat anti-rabbit antibody (1:2000), and after washing for 1 h with PBS either analyzed by the colour-test (substrates of alkaline phosphates, NBT and BCIP), or a highly sensitive luminescence technique (ECL, CDP-star, Tropix) was applied to detect immunoreactive spots on membranes.

Statistical analysis

Determination of standard deviation (SD) for dry weight (growth of *Heliscus*) and protein spots (Hsp70, metallothioneins) was performed with MS Excel 6.0. Significant differences of these data were calculated using Student's *t*-test. P values <0.05 were considered significant (Timischl, 2000).

Results

Comparison of aquatic hyphomycete *Heliscus lugdunensis* strains Hl-BB and Hl-H4 showed significant differences in their growth and the induction of Hsp70 and metallothionein-like proteins by cadmium.

At first, the strains were studied in liquid culture for their response to Cd or heat stress, using Hsp70-induction as a bioindicator. Seven days old cultures were incubated for 4 or 24 h at 14 °C in the presence of 40, 80 or 120 µM $CdCl_2$. Thereafter, cytosolic proteins were isolated and analyzed by SDS-PAGE and immunoblotting, using a polyclonal Hsp70-antibody. The results are presented in Fig. 1. In Hl-BB, a very low level of Hsp70 under the conditions tested was observed. In Hl-H4, however, concentrations in the range of 40–120 µM Cd produced a clear expression of Hsp70. The highest Hsp70 expression was registered with 80 µM Cd after 24 h incubation, yet a 4 h treatment with 120 µM Cd also resulted in an elevated Hsp70 synthesis. A prolonged treatment with 120 µM Cd caused no Hsp70-induction, probably because of toxic effects.

This remarkable difference in Cd-response of both strains was illustrated by a growth-inhibitory test in the presence of 80 µM Cd. Upon 12 days incubation, the growth of strain Hl-BB (as evaluated by the dry mass of mycelia) decreased to 40% compared to control culture (without Cd), while the growth of strain Hl-H4 reached 86% under the same conditions (Fig. 2).

Both strains responded to heat stress in a similar way, as with Cd. 7 d-Cultures of Hl-BB and Hl-H4 were incubated for 2–4 h at 40° and the cytosolic proteins were analyzed as above. In Hl-BB, only a marginal presence of Hsp70 was registered. In contrast, in Hl-H4 a pronounced expression of the stress protein was evident, especially after 2 h of heat stress (Fig. 3). Upon prolonged heating (4 h), the amount of Hsp70 decreased, probably because of damaging effects on mycelia.

When subjecting the strains to a double stress, first to Cd, and then to heating, the response remained the same: a marginal induction in Hl-BB, and a definite expression

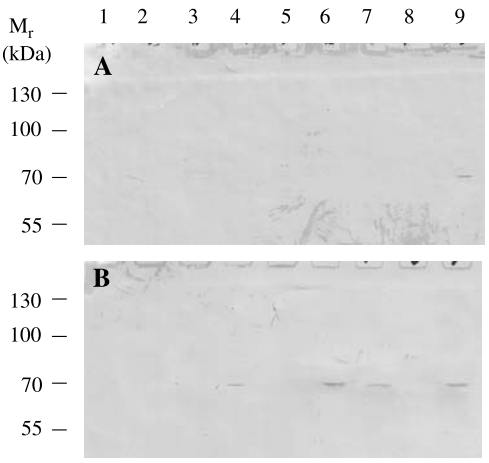

Fig. 1. Induction of Hsp70 in *H. lugdunensis* strains Hl-BB and Hl-H4 by cadmium. The mycelia of Hl-BB (**A**) and Hl-H4 (**B**) were cultivated 7 d at 14 °C (control, *2*), then incubated for 4 h (*3*) or 24 h (*4*) at 40 µM Cd, for 4 h (*5*) or 24 h (*6*) at 80 µM Cd, and for 4 h (*7*) or 24 h (*8*) at 120 µM Cd. *9* Illustrates the serological result with 2 µg of *Cucurbita* Hsp70. Buffer soluble proteins were extracted, 20 µg were subjected to each lane, separated by 10% Laemmli-SDS-PAGE, transferred to NC-membrane and incubated with Hsp70-antibody. *1* Fermentas marker proteins, 11–170 kDa. Electrophoregramms after analysis by phosphatase-colour-test are depicted

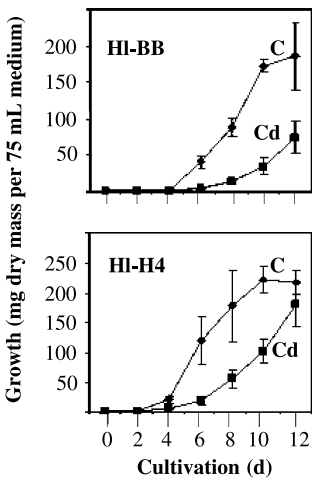

Fig. 2. Growth of *H. lugdunensis strains* Hl-BB and Hl-H4 without (*C*, control) or in the presence of cadmium (*Cd*, 80 µM) in the time range of 12 days. *Cd* was added at beginning of the experiment

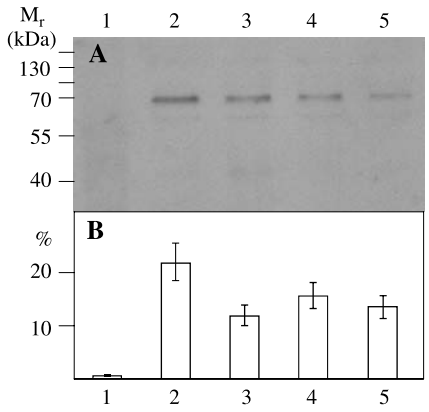

Fig. 3. Induction of Hsp70 in *H. lugdunensis* strains Hl-BB and Hl-H4 upon heat stress. **A** Electrophoresis after analysis by ECL-test. **B** Semi-quantitative calculation of spot intensity. The mycelia of two strains were cultivated for 7 d at 14 °C without (control) or with 80 µM Cd, added at 4th day. Then, control- and Cd-treated mycelia were incubated for 2 or 4 h at 40 °C. Buffer soluble proteins were extracted, 20 µg were subjected to each lane and separated by 10–17% Laemmli-SDS-PAGE: Strain Hl-BB upon 2 or 4 h at 40 °C (*1*). Note that similar results were obtained upon heating of Cd-treated Hl-BB; strain Hl-H4 upon 2 or 4 h at 40 °C (*2* and *4*); Cd-treated Hl-H4 upon 2 or 4 h at 40 °C (*3* and *5*). Control strains exhibited similar Hsp70-expression, as that in *1*

of Hsp70 in Hl-H4 (Fig. 3). It should be mentioned that the Hsp70 levels in this case were lower compared to those upon heat stress only, due to the toxic effect of the combined treatment.

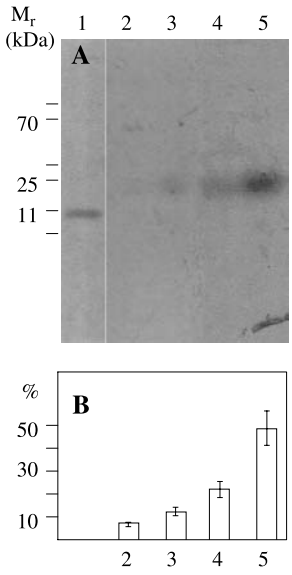

Fig. 4. Induction of sulphur-rich proteins in *H. lugdunensis* strain Hl-BB by cadmium. **A** Electrophoresis after analysis by UV-light. **B** Semi-quantitative calculation of spot intensity. The strain was cultivated for 7 d at 14 °C by shaking and then incubated for 24 h without or with cadmium. Proteins were extracted and each of 20 µg protein was separated on 12–20% Schägger-gradient gel: Monobromobimane-derivative of 5 µg MT-1 (rabbit, see Materials and methods) (*1*); control of shaking culture (*2*); 40, 80 and 120 µM Cd (*3–5*)

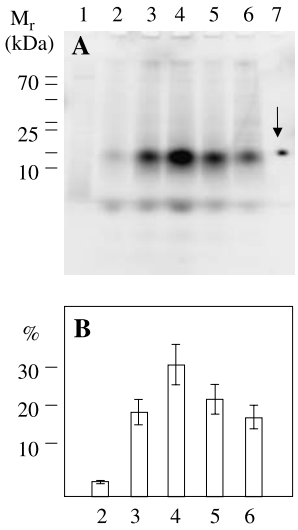

Fig. 5. Induction of sulphur-rich proteins in *H. lugdunensis* strain Hl-H4 by cadmium. **A** Electrophoresis after analysis by UV-light. **B** Semi-quantitative calculation of spot intensity. The material was cultivated for 7 d at 14 °C by shaking and then incubated for 24 h without or with cadmium. Proteins were extracted and each of 20 µg protein was separated on 12–20% Schägger-gradient gel: Fermenta-marker SM1811, 9 recombinant proteins 10–250 kDa, 5 µl (*1*); control of shaking culture (*2*); 40, 80 and 120 µM Cd (*3–5*); control of a fixed culture (*6*); monobromobimane-derivative of 2 µg MT-1 (rabbit) at 11 kDa (arrow) (*7*)

Secondly, protein fractions were isolated, thiol groups of proteins reacted with monobromobimane and then, the labelled proteins were separated by SDS-PAGE. A high fluorescence of bimane-derivatives of thiol-rich proteins was only detected (Figs. 4, 5). *Heliscus* cells of these two strains responded to cadmium over 24 hours treatment by a different concentration-dependent induction of sulfur-rich metallothioneins or metallothionein-like proteins. Thus, with Hl-BB a maximum MTLP-induction was obtained at 120 µM Cd reaching about 600% of that of controls (being 100%) (Fig. 4A, B, lane 5 compared to lane 2, control), whereas with Hl-H4 a maximal protein induction was produced at 80 µM Cd reaching about 800% of control levels (Fig. 5A, B, lane 4 compared to lane 2, control). Surprisingly, the relative molecular masses of MTLPs analyzed by SDS-PAGE were 20 and 11 kDa in the strains Hl-BB and Hl-H4, respectively (Figs. 4A, 5A). Further unknown fluorescent spots at about 5 kDa, and very small ones at about 2 kDa were detected (Fig. 5). No significant increase of MTLPs was observed by heating of 7 days old mycelia for 4, 8 or 24 h at 40 °C (not shown).

Discussion

The mechanisms of heavy metal adaptation and tolerance in filamentous fungi are not well understood. Current

Table 1. Induction of Hsp70 in *H. lugdunensis* strains from polluted and unpolluted areas

Induction agent	Concentration (μM)	Time of treatment (h)	Hl-BB	Hl-H4
Heat (40°)		2	±	++
Cd	40	1	–	–
		2	–	–
		4	–	–
		24	–	±
	80	1	–	–
		4	–	
		24	toxic	++
	120	1	–	+
		4	toxic	+
		24	toxic	toxic
	IC_{50}[a]	120	45[b]	100[c]

++ Strong positive
+ Positive
± Low induction
– Negative
[a] Inhibitory concentration reducing the growth of fungi by 50%
[b] Miersch et al. (1997)
[c] Jaeckel et al. (2005a)

evidence suggests that stress proteins may play an important role in this process, yet their detailed functions remain to be elucidated. Special attention in this respect is paid to metallothionein-like proteins (MTLPs) and heat-shock proteins (Hsp). In this work, we were concerned with the induction of Hsp70 and some MTLPs in *H. lugdunensis* strains Hl-H4 and Hl-BB, growing on differently polluted natural habitats, in order to correlate their expression with heavy metal resistance of the strains. In vitro studies have revealed a significantly higher resistance of Hl-H4 towards Cd (IC50 of 100 μM) (Jaeckel et al., 2005a) as compared to Hl-BB (IC50 of 45 μM) (Miersch et al., 1997). An elevated resistance of Hl-H4 to Cd is also shown in this study (Fig. 2). We demonstrate that upon Cd-stress, Hsp70 are actively synthesized in the strain Hl-H4 isolated from a highly polluted site, and to a much lower degree (trace) in Hl-BB growing on a non-contaminated area. The Hsp70-expression, as summarized in Table 1, was time and dose-dependent, reaching a maximum after 24 h at 80 μM Cd. This acquired Hsp70 response was further evidenced by subjecting both strains to heat (40 °C). Again, there was a strong Hsp70-induction in Hl-H4, and only a marginal one in Hl-BB. This remarkable feature of Hl-H4 merits special attention. Transcription of Hsp70 gene, known to be activated by heat stress, is also induced by a variety of metals such as Zn, Cd, Cu and Hg (Williams and Morimoto, 1990; Murata et al., 1999). It has been shown that the induction of Hsp70 by heavy metals, as well as by heat shock, is mediated by metal-activated HSF1 (heat shock factor 1) (Mosser et al., 1988; Williams and Morimoto, 1990; Murata et al., 1999). We assume that under continuous heavy metal exposure, Hl-H4 has acquired a higher steady-state activation ability of HSF1 compared to Hl-BB, thus ensuring a better survival. To our knowledge, this is the first report to show the involvement of Hsp70 in the adaptive response of aquatic hyphomycetes to heavy metal toxicity. The majority of studies on Hsp70 in fungi have been performed on ascomycetes such as *Saccharomyces cerevisiae* (Boorstein et al., 1994), and some other fungal phyla including zygomycete *Rhizopus nigricans*, basidomycetes *Boletus edulis* and *Cryptococcus curvatus*, and chytridiomycete *Blastocladiella emersonii* (Cernila et al., 2003). The authors report elevated Hsp70-mRNA content in *R. nigricans* upon heat or Cu-stress (Cernila et al., 2000), or elevated Hsp70 concentrations in *B. edulis* after Cd-exposure (Collin-Hansen et al., 2005), the effects being in all cases considered as immediate stress and/or defence response (but no adaptation) against metal toxicity.

Hl-H4 and Hl-BB exhibit specific, environmentally related induction of sulphur-rich proteins, MTs or MTLPs, upon Cd-treatment, differing in molecular mass and induction pattern, as unambiguously shown by the highly sensitive bimane derivatisation method of SH-containing proteins (Fan et al., 2004). Hl-H4 responded by a strong expression of 11 kDa SH-rich protein (most likely metallothionein). The cadmium-inducibility of 11 kDa MT in Hl-H4 only suggests its involvement in metal tolerance and detoxification. Remarkable is the similarity of the induction pattern of this metallothionein to that of Hsp70 (both stress proteins reached maximum expression at 80 μM Cd) (compare Figs. 1 and 5), which suggests interdependence of their induction mechanisms and roles in metal resistance. Recently, a cross-talk of heat shock and heavy metal stress pathways in human cell has been suggested (Uenishi et al., 2006). On the contrary, Hl-BB reacted to Cd-stress by a weak induction of SH-rich protein of about 20 kDa (not expressed in Hl-H4). The induction pattern of this MT (Fig. 4) differed completely from that of 11 kDa MT or Hsp70, pointing rather to an immediate stress bioindication than detoxification in Hl-BB.

MTs are ubiquitous, functionally diverse metal-binding proteins of relatively low molecular mass that play major roles in metal homeostasis and/or detoxification (Kägi and Schaffer, 1988; Cobett and Goldsbrough, 2002; Roosens et al., 2005). Fungi contain mainly small MTs (3–6 kDa). In aquatic fungi, only a few of them have been identified so far to be involved in metal detoxification.

The content of thiol-rich proteins was reported to increase significantly in the aquatic hyphomycetes *Fontanospora fusiramosa* and *Flagellospora curta*, exposed to Cd (Guimaraes-Soares et al., 2007). Recently, 2.3 kDa MT has been identified and characterized in *H. lugdunensis* strain H8-2-1, isolated from a moderately polluted site (Jaeckel et al., 2005b). In other phyla, MT of 53 amino acid residues has been found in certain mutants of *S. cerevisiae* to produce copper or cadmium resistance (Tohoyama et al., 1995). Small copper binding MTs were described for the ectomycorrhizal fungi *Laccaria laccata* and *Paxillus involutus* (Howe et al., 1997). In *P. involutus*, Cd has been found to induce 3 kDa MT-related proteins, thought to be involved in metal tolerance (Courbot et al., 2004). Class II MTs were induced by Cu- and Cd-exposure in the heavy metal resistant ascomycete *Beauveria bassiana* (Kameo et al., 2000). Our article is the first to describe MT of about 11 kDa involved in adaptation of aquatic hyphomycetes to continuous heavy metal exposure, thus expanding the knowledge on the function of diverse MTs. Interestingly, 11 kDa Cd-binding metallothionein-like protein from the unicellular green algae *Chlorella sorokiana* has been reported to confer an extremely high resistance of this species to heavy metals (Yoshida et al., 2006). Other sulphur-rich proteins between 70 and 140 amino acids, such as hydrophobins (Temple and Horgen, 2000) were not found in aquatic fungi.

In conclusion, the specific induction of Hsp70 and/or distinct MTs in the range of 11 kDa in *Heliscus* strain Hl-H4 appear to be essential adaptive mechanisms to continuous heavy metal exposure.

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References

Bärlocher F (1987) Aquatic hyphomycetes spora in ten streams of New Brunswick and Nova Scotia. Can J Bot 65: 76–79

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8: 93–99

Boorstein WR, Ziegelhoffer T, Craig EA (1994) Molecular evolution of the Hsp70 multigene family. J Mol Evol 38: 1–17

Braha B, Tintemann H, Krauss G, Ehrman J, Bärlocher F, Krauss G-J (2007) Stress response in two strains of the aquatic hyphomycetes *Heliscus lugdunensis* after exposure to cadmium and copper ions. BioMetals 20: 93–105

Cernila B, Cresnar B, Breskvar K (2000) Isolation, partial length sequences and expression of steroid inducible Hsp70 gene from *Rhizopus nigricans*. Pflugers Arch 439 [Suppl 3]: R97–R99

Cernila B, Cresnar B, Breskvar K (2003) Molecular characterization of genes encoding Hsp70s in the zygomycete *Rhizopus nigricans*. Cell Stress Chap 8: 317–328

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. Ann Rev Plant Physiol Plant Mol Biol 53: 159–182

Collin-Hansen C, Andersen RA, Steines E (2005) Molecular defense systems are expressed in the king bolete (*Boletus edulis*) growing near metal smelters. Mycologia 97: 973–983

Courbot M, Diez L, Ruotola R, Chalot M, Leroy P (2004) Cadmium-responsive thiols in the ectomycorrhizal fungus *Paxillus involutus*. Appl Environ Microbiol 70: 7413–7417

Fan TW-M, Lane AN, Higashi RM (2004) An electrophoretic profiling method for thiol-rich phytochelatins and metallothioneins. Phytochem Anal 15: 175–183

Fomina MA, Burford EP, Gadd GM (2005) Toxic metals and fungal communities. In: Dighton J, White JF, Oudemans P (eds) The fungal community, its organization and role in the ecosystem. Taylor and Francis, Boca Raton London New York Singapore, pp 733–758

Gadd G (2001) Metal transformations. In: Gadd GM (ed) Bioremediation. Cambridge University Press, Cambridge, pp 359–382

Guimaraes-Soares L, Pascoal C, Cassio F (2007) Effects of heavy metals on the production of thiol compounds by the aquatic fungi *Fontanospora fusiramosa* and *Flagellospora curta*. Ecotoxicol Environ Saf 66: 36–43

Howe R, Evans RL, Ketteridge SW (1997) Copper binding proteins in ectomycorrhizal fungi. New Phytol 135: 123–131

Jaeckel P, Krauss G-J, Krauss G (2005a) Cadmium and zinc response of the fungi *Heliscus lugdunensis* and *Verticillium* cf. *alboatrum* isolated from highly polluted water. Sci Total Environ 346: 274–279

Jaeckel P, Krauss G, Menge S, Schierhorn A, Rücknagel P, Krauss G-J (2005b) Cadmium induces a novel metallothionein and phytochelatin 2 in an aquatic fungus. Biochem Biophys Res Commun 333: 150–155

Jarosz-Wilkolazka A, Graz M, Braha B, Menge S, Schlosser D, Krauss G-J (2005) Species-specific Cd-stress response in the white rot basiomycetes *Abortiporus biennis* and *Cerrena unicolor*. BioMetals 19: 39–49

Kägi JH, Schaffer A (1988) Biochemistry of metallothionein. Biochemistry 27: 8509–8515

Kameo S, Iwahashi H, Kojima Y, Satoh H (2000) Induction of metallothioneins in the heavy metal resistant fungus *Beauveria bassiana* exposed to copper or cadmium. Analusis 28: 382–385

Krauss G, Bärlocher F, Schreck P, Kranich W, Miersch J, Dermitzel J, Wennrich R, Krauss G-J (1998) Aquatic hyphomycetes at extremely polluted sites in the Mansfelder Land area. In: Becker PM (ed) Microbiology of polluted aquatic ecosystems, UFZ-Series 10. UFZ Centre for Environmental Research, Leipzig-Halle, pp 76–83

Krauss G, Bärlocher F, Schreck P, Wennrich R, Gläser W, Krauss G-J (2001) Aquatic hyphomycetes occur in hyperpoluted waters in Central Germany. Nova Hedwigia 72: 419–428

Krauss G, Schlosser D, Krauss G-J (2005a) Aquatic fungi in heavy metal and organically polluted habitats. In: Desmukh SK, Rai MK (eds) Biodiversity of fungi: their role in human life. Oxford & IBH Publishing Co. Pvt. Ltd., New Dehli, India and Sci. Publ. Inc., Enfield, NH, USA, pp 221–246

Krauss G-J, Krauss G, Menge S, Wesenberg D (2005b) Cadmium stress response at the thiol peptide level in mosses and fungi. In: Saito K, DeKok LJ, Sulen I, Hawkesford MJ, Schnuck E, Sirko A, Renneberg H (eds) Sulfur transport and assimilation in plants in the post genomic era. Backhuys Publ., Leiden, pp 233–236

Laemmli K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lowry DH, Rosebrough NJ, Farr AL, Randall RL (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193: 265–275

Miersch J, Bärlocher F, Bruns I, Krauss G-J (1997) Effects of cadmium, copper, and zinc on growth and thiol content of aquatic hyphomycetes. Hydrobiologia 346: 77–84

Mosser DD, Theodorakis NG, Morimoto RI (1988) Coordinate changes in heat shock element-binding activity and Hsp70 gene transcription rates in human cells. Mol Cell Biol 8: 4736–4744

Murata M, Gong P, Suzuki K, Koizumi S (1999) Differential metal response and regulation of human heavy metal-inducible genes. J Cell Physiol 180: 105–113

Neumann D, zur Nieden U, Manteufel R, Walter G, Scharf K-D, Nover L (1987) Intracellular localization of heat shock proteins in tomato cell cultures. Eur J Cell Biol Chem 43: 71–81

Roosens NH, Leplae R, Bernard C, Verbruggen N (2005) Variations in plant metallothioneins: the heavy metal hyperaccumulator *Thlaspi caerulescens* as a study case. Planta 222: 716–729

Schägger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166: 368–379

Sridhar KR, Bärlocher F, Krauss G-J, Krauss G (2005) Response of aquatic hyphomycete communities to changes in heavy metal exposure. Internat Rev Hydrobiol 90: 21–32

Temple B, Horgen PA (2000) Biological role for cerato-ulmin, a hydrophobin secreted by the pathogen, *Ophistoma ulmi* and *O. novo-ulmi*. Mycologia 92: 1–9

Timischl W (2000) Biostatistik. Eine Einführung für Biologen und Mediziner. Springer, Berlin Heidelberg New York Tokyo, pp 101–106

Tohoyama H, Inouhe M, Joho M, Murayama T (1995) Production of metallothionein in copper- and cadmium-resistant strains of *Saccharomyces cerevisiae*. J Ind Microbiol 14: 126–131

Uenishi R, Gong P, Suzuki K, Koizumi S (2006) Cross talk of heat shock and heavy metal regulatory pathways. Biochem Biophys Res Commun 341: 1072–1077

Vepachedu SKVRR, Akthar N, Mohan PM (1997) Isolation of a cadmium tolerant *Curvularia* sp. from polluted effluents. Curr Sci 73: 453–455

Wang W, Vinocur B, Shoseyov O, Altman A (2004) The role of plant heat-shock proteins/molecular chaperones in the abiotic stress response. Trends Plant Sci 9: 244–252

Weber K, Osborne M (1969) The reliability of the molecular weight determination by dodecyl sulfate polyacrylamide gel electrophoresis. J Biol Chem 244: 4406–4412

Williams GT, Morimoto RI (1990) Maximal stress-induced transcription from the human Hsp70 promoter requires interactions with the basal promoter elements independent of rotational alignment. Mol Cell Biol 10: 3125–3136

Yoshida N, Ishii K, Okuno T, Tanaka K (2006) Purification and characterization of cadmium-binding protein from unicellular alga *Chlorella sorokinian*. Curr Microbiol 52: 460–463

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