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Species-specific Cd-stress response in the white rot basidiomycetes *Abortiporus biennis* and *Cerrena unicolor*

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Abstract

The effect of cadmium (Cd) on fungal growth, Cd bioaccumulation and biosorption, and on the formation of potential heavy metal response indicators such as thiols, oxalate, and laccase was investigated in the white rot fungi *Cerrena unicolor* and*Abortiporus biennis*. Only the highest Cd concentration employed (200 μM) inhibited growth of *C. unicolor*, whereas already lower Cd concentrations caused decreasing mycelia dry weights in *A. biennis*. Cd biosorption onto the mycelial surface was the predominant Cd sequestration mechanism in *C. unicolor*. Surface-bound and bioaccumulated Cd concentrations were essentially in the same range in *A. biennis*, leading to considerably higher intracellular Cd concentrations in *A. biennis* than in *C. unicolor*. Oxalate and laccase were produced by both of the fungal strains and their extracellular levels were elevated upon Cd exposure. Oxalate concentrations and laccase titres were considerably higher in *C. unicolor* than in *A. biennis*. Both fungi responded to increasing Cd concentrations by increasing intracellular amounts of thiol compounds (cysteine, γ-glutamylcysteine, glutathione in both its reduced and oxidized form) but Cd application increased the amounts of thiols to a higher extend in *A. biennis*. Taken together, these species-specific responses towards Cd suggest that *C. unicolor* possesses a more efficient system than *A. biennis* to keep intracellular Cd concentrations low.

Introduction

Fungi are ubiquitous in the natural environment and play important roles in the decomposition of organic matter and nutrient cycling. Furthermore, they can affect the formation of soluble and insoluble forms of toxic metals, as well as volatile metal derivatives, and thus influence metal distribution in the environment. There has been renewed interest in the effects of metals on fungi because of their potential application in bioremediation (Gadd & Sayer 2000; Gadd 2001).

Fungi can remove heavy metals from environmental compartments through either bioaccumulation or biosorption. In bioaccumulation, metals are transported from the outside of the microbial cell through the cellular membrane into the cytoplasm, where the metal is sequestered and therefore immobile. Biosorption is a term designated for all of the passive interactions with metals whereas positively charged metal ions are sequestered through metal adsorption to the negative ionic groups on cell surfaces (Palmisano & Hazen 2003).

Physiological responses to metal contamination in fungi indicate several systems of active or passive defence that can help them to resist or to reduce metal toxicity (Gadd 2001; Baldrian 2003; Krauss *et al*. 2004). Binding of metals to cell walls, and their sequestration by binding to thiol peptides or organic acids and complexation in vacuoles could decrease the toxic effects of free metal ions by effectively lowering their intracellular concentrations (Gadd & Sayer 2000; Krauss *et al*. 2003, 2004). These tolerance mechanisms are not well understood in wood-rotting fungi and most data obtained so far concern yeasts (Inouhe *et al*. 1996), aquatic fungi (Krauss *et al*. 2004), and mycorrhizal fungi (Blaudez *et al*. 2000; Jacob *et al*. 2004). An important role in intracellular metal detoxification in fungi and plants is attributed to metal binding by cysteine-rich peptides such as reduced glutathione (GSH; γ-Glu–Cys–Gly), phytochelatins (PCs), or metallothioneins (MTs) (Cobbett & Goldsbrough 2002). GSH possesses multiple functions and is involved in, e.g., antioxidative defence, cell proliferation, and chelation of metal ions (Penninckx 2002; Schützendübel & Polle 2002). It is further the direct precursor of glutathione-related PCs [$(\gamma$-Glu–Cys$)_n$–Gly, where n = 2–11] also reported for filamentous fungi (Cobbett & Goldsbrough 2002; Gadd 2001; Miersch *et al*. 2001; Münger & Lerch 1985). Whereas the bioinduction of cysteine-rich peptides in yeasts, filamentous fungi, some basidiomycetes, and ectomycorrhizal fungi has been demonstrated, to date only very limited information is available about heavy metal-detoxifying principles in wood-rotting fungi (Münger & Lerch 1985; Gadd 2001). The understanding of the mechanisms governing heavy metal resistance in wood-rotting fungi is of considerable interest because of their significance for natural ecosystem functions and their potential applicability in biotechnological decontamination processes. Organic acids such as oxalate have frequently been observed in wood-rotting basidiomycetes (Shimada *et al*. 1997). The reported formation of oxalate crystals by wood-rotting fungi response to toxic metal concentrations suggests a role in fungal detoxification of metals (Jarosz-Wilkołazka & Gadd 2003). White rot fungi produce extracellular ligninolytic enzymes such as laccases and various peroxidases (Leonowicz *et al*. 1999). Laccases (E.C. 1.10.3.2) are multicopper containing oxidoreductases that contribute to the biodegradation of lignin and organic xenobiotics. Furthermore, these enzymes are believed to be involved in the fungal synthesis of melanins (Fogarty & Tobin 1996; Baldrian 2003). Fungal melanins have been shown to efficiently adsorb heavy metals, and accordingly laccases may contribute to the fungal defence against toxic metals (Rizzo *et al*. 1992; Fogarty & Tobin 1996).

The aim of this paper was to compare the Cd resistance, Cd bioaccumulation and biosorption, as well as the effect of Cd on the formation of potential heavy metal response indicators such as different thiol compounds, oxalate, and laccase in two strains of the white rot fungi *Cerrena unicolor* and *Abortiporus biennis*. We selected these strains because of their different sensitivities towards various stress conditions as previously described (Jarosz-Wilkołazka *et al*. 1998; Fink-Boots *et al*. 1999).

Materials and methods

Fungal strains and culture conditions

Cerrena unicolor (Bull. ex Fr.) Murr and *Abortiporus biennis* (Bull. ex Fr.) Singer were obtained from the culture collection of the Department of Biochemistry, Maria Curie-Skłodowska University, Lublin, Poland, and maintained on 2% (w/v) malt agar plates. For liquid culture experiments, mycelia-containing agar plugs were added to the liquid medium described below (about 0.5 cm^2 surface per ml of medium) and homogenized, using dispersing devices. After inoculation with 4% (v/v) of the homogenate, stationary cultures were incubated in 100-ml Erlenmeyer flasks containing 50 ml Lindeberg medium (Lindeberg & Holm 1952) at 28 °C. Cd was added in the form of $CdCl_2$ to 6-day-old cultures at concentrations of 10, 50, 100, or 200 μM, respectively. The mycelia were harvested by filtration through Whatman GF/A filter papers at 2, 4, 6, and 8 days after $CdCl_2$ addition and stored at −80 °C.

Determination of biosorbed and bioaccumulated Cd

After incubation with $CdCl_2$, mycelia were subsequently washed 5 times with 5 mM

ethylenediaminetetraacetic acid (EDTA) to remove superficially bound metals (Jarosz-Wilkołazka *et al*. 2002). The Cd content of the washing solution was measured by atomic absorption spectroscopy as described below, and the amount of Cd biosorbed onto mycelial surfaces was calculated from the Cd content in the washing solution. After this, fungal mycelia were dried and digested in a microwave system (CEM, MDS 2100) (Bruns *et al*. 1997), and assessed for their intracellular Cd contents. Cd concentrations were determined in a Carl Zeiss Solar Unicam 929 atomic absorption spectrophotometer. The instrument was calibrated with Cd standard solutions.

Determination of extracellular oxalate using capillary zone electrophoresis

Capillary zone electrophoresis (CZE) analyses were performed on a Thermo Capillary Electrophoresis, Crystal 100 (Thermo Separation Products). Separations were carried out using a fused silica capillary with a total length of 75 cm (50 cm to detection window) and an inner diameter of 50 μm. The applied voltage was −25 kV, and the capillary temperature was maintained at 25 °C. Samples were injected hydrodynamically for 1 s. Indirect detection was operated at 210 nm. Buffer solution was prepared by dissolving 41.3 mg of phthalic acid and 4.8 mg cetyltrimethylammonium bromide (CTAB) in a mixture of 0.25 ml methanol, 0.4 ml 1 N NaOH, and 5 ml MilliQ water. The pH of the solution was adjusted to 7.0 with NaOH, and the total volume was brought to 50 ml (Galkin *et al*. 1998).

Determination of laccase activity

Laccase (EC 1.10.3.2) activity was measured by monitoring the oxidation of syringaldazine according to Leonowicz and Grzywnowicz (1983). The reaction mixture contained 0.025 mM syringaldazine and enzyme samples in 50 mM citrate-phosphate buffer (pH 5.2). The oxidation of syringaldazine was monitored by the increase in the A_{520} at 30 °C. The activity was calculated from an oxidation product extinction coefficient of 65 000 M^{-1} cm^{-1} and expressed as mU ml^{-1}, where 1 U = 1 μmol of product formed per min.

Determination of thiol compounds

For determination of non-protein thiol compounds such as the glutathione precursors cysteine (Cys) and γ-glutamylcysteine (γ-EC), as well as GSH and its oxidation product (GSSG), 50 mg of fungal material frozen in liquid nitrogen was disintegrated and extracted with 1000 μl of a 0.1 N HCl solution containing 80 mg of polyvinylpolypyrrolidone (PVP) for 15 min and subsequently centrifuged for 10 min at 14 000 rpm and 4 °C. An aliquot of the supernatant (120 μl) was supplemented 180 μl of a 0.2 N 2-(cyclohexylamino)ethanesulfonic acid (CHES) solution (pH 9.3) and 30 μl of 30 mM dithiothreitol (DTE), and incubated for 1 h on ice. GSH, γ-EC, and cysteine were analysed after derivatization with monobromobimane (MBB). Derivatization was performed by adding 10 μl of a 30 mM MBB solution and subsequent incubation for 15 min in the dark, and terminated by adding 250 μl of a 5% (w/v) acetic acid solution. To determine GSSG, GSH in supernatants was masked by adding 50 μl of a 5 mM N-ethylmaleimide (NEM) solution. Excess NEM was extracted with toluene. The remaining GSSG was reduced with 30 μl of 30 mM DTE, allowed to react for 1 h, and subsequently derivatized with MBB as already described. After centrifugation, thiol compounds were separated with a Merck-Hitachi high-performance liquid chromatography (HPLC) system, equipped with a Lichrospher 60 RP select B silicagel column (Merck-Hitachi). Elution was carried out with a linear gradient of 10% (v/v) to 90% methanol in water (pH 4.0, adjusted with acetic acid) at a flow rate of 0.9 ml min^{-1}. The derivatization product was detected by fluorescence (420 nm for excitation and 520 nm for emission) (Miersch *et al*. 1997).

For determination of PCs, 100 mg of fungal material frozen in liquid nitrogen was disintegrated and extracted with 200 μl of 1 N NaOH containing 0.2 mg of $NaBH_4$ for 15 min, and subsequently centrifuged for 5 min at 14 000 rpm and 4 °C. An aliquot of the supernatant (100 μl) was supplemented with 28 μl of 3.6 N HCl, incubated for 15 min on ice, centrifuged for 5 min at 14 000 rpm and 4 °C, and immediately assayed by HPLC as previously described (Miersch *et al*. 2001). Thiol-containing peptides were determined upon post-column derivatization, using

5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; Ellman reagent) together with GSH and PCs as standards.

Determination of fungal dry weights

Fungal mycelia were dried to a constant mass for 4 h at 80 °C, and dry weights were determined with a balance.

Results

Effect of Cd on the growth and morphology of fungal mycelia

With the exception of the highest Cd concentration employed, which decreased the fungal dry weight after 6 days of Cd exposure by approximately 30%, addition of Cd did not remarkably affect the mycelial dry weight of *C. unicolor* cultures (Table 1). Cd was obviously more toxic to *A. biennis*, where increasing Cd concentrations clearly caused decreasing fungal dry weights (Table 1). The morphology of mycelia of both examined strains remarkably differed from each other. Addition of Cd ions to *C. unicolor* cultures resulted in the development white, aerial, and very dense hyphae. In contrast, during an 8-day Cd exposure the mycelium of *A. biennis* became very fragile. Also, *A. biennis* cultures growing in the presence of Cd produced an orange–brown pigment that coloured both, the mycelium as well as the cell-free culture medium.

Uptake and distribution of Cd in fungal mycelia

The two examined strains remarkably differed in their Cd bioaccumulation and biosorption behaviour. Cd biosorption onto the surface of mycelium was the predominant mechanism of Cd fixation in *C. unicolor*, where the concentrations of biosorbed Cd were about one order of magnitude higher than those of accumulated Cd (Table 2). In *A. biennis*,

Table 1. Effect of $CdCl_2$ addition on mycelia dry weights of *C. unicolor* and *A. biennis* cultures after 2 and 6 days of incubation in presence of $CdCl_2$. Data represent means ± SD ($n = 3$).

$CdCl_2$(μM)	Fungal dry weight (mg/ml) [% of control]			
	C. unicolor		*A. biennis*	
	2 days	6 days	2 days	6 days
0 (control)	6.15 ± 0.64 (100)	23.05 ± 3.46 (100)	8.05 ± 0.35 (100)	17.40 ± 2.12 (100)
10	7.10 ± 0.82 (115)	23.03 ± 4.82 (100)	6.47 ± 1.63 (80)	13.47 ± 2.55 (77)
50	6.27 ± 1.15 (102)	21.63 ± 5.19 (94)	7.10 ± 0.36[a] (88)	11.17 ± 1.50[a] (67)
100	5.83 ± 1.17 (95)	21.77 ± 2.20 (94)	5.87 ± 1.40 (73)	10.73 ± 2.66[a] (62)
200	6.97 ± 1.69 (113)	16.10 ± 2.96 (70)	5.67 ± 0.15[a] (70)	9.43 ± 2.78[a] (54)

[a]Significantly lower ($P < 0.05$) than the corresponding control value, as obtained by Student's t test.

Table 2. Biosorption and bioaccumulation of cadmium ions by *C. unicolor* and *A. biennis* cultures after 2 days of incubation in presence of $CdCl_2$. Data represent means ± SD ($n = 3$).

$CdCl_2$ (μM)	Biosorption (μmol Cd/g dry mycelium)		Bioaccumulation (μmol Cd/g dry mycelium)	
	C. unicolor	*A. biennis*	*C. unicolor*	*A. biennis*
10	2.43 ± 0.29[a, b]	0.21 ± 0.04	0.20 ± 0.11	0.29 ± 0.11
50	4.88 ± 1.34[a, b]	1.99 ± 0.22[a]	0.73 ± 0.08	0.94 ± 0.16
100	10.16 ± 1.90[a, b]	4.43 ± 0.53[a]	1.09 ± 0.13[c]	2.69 ± 0.27
200	16.05 ± 1.82[a, b]	5.53 ± 0.85	0.66 ± 0.04[c]	6.39 ± 0.94

[a]Significantly higher ($P < 0.05$) than the corresponding bioaccumulation value, as obtained by Student's t test.
[b]Significantly higher ($P < 0.05$) than the corresponding biosorption value of *A. biennis*.
[c]Significantly lower ($P < 0.05$) than the corresponding bioaccumulation value of *A. biennis*.

both surface-bound and bioaccumulated Cd concentrations increased upon exposure to increasing amounts of Cd and were essentially in the same concentration range at a given amount of Cd (Table 2). Accordingly, bioaccumulation of Cd was significantly higher in *A. biennis* than in *C. unicolor* at 100 and 200 μM Cd.

Effect of Cd on the secretion of oxalate

Oxalate was produced by both of the fungal strains and extracellular oxalate concentrations were clearly elevated upon Cd exposure (Figure 1). *C. unicolor* secreted higher concentrations of oxalate than *A. biennis* (up to approximately 5–6 mM in the presence Cd and about 4 mM without Cd addition) that were essentially maintained until the end of cultivation. In *A. biennis*, all Cd concentrations employed led to an increase in the respective oxalate concentrations until day 4 after Cd addition, with the highest oxalate concentration of approximately 0.3 mM being observed in presence of 10 and 50 μM Cd, respectively. Later on the oxalate concentrations dramatically decreased for all examined Cd concentrations, possibly due to toxic effects of Cd on the metabolism of *A. biennis*.

Effect of Cd on extracellular laccase activities

All Cd concentrations employed stimulated extracellular laccase activities in *C. unicolor* and *A. biennis* (Figure 2). In *C. unicolor*, elevation of extracellular laccase activity was essentially saturated already at 50 μM Cd, whereas the maximum Cd concentration applied (200 μM) still caused a slight increase in laccase activity up to the day 6 after Cd addition in *A. biennis*. Generally,

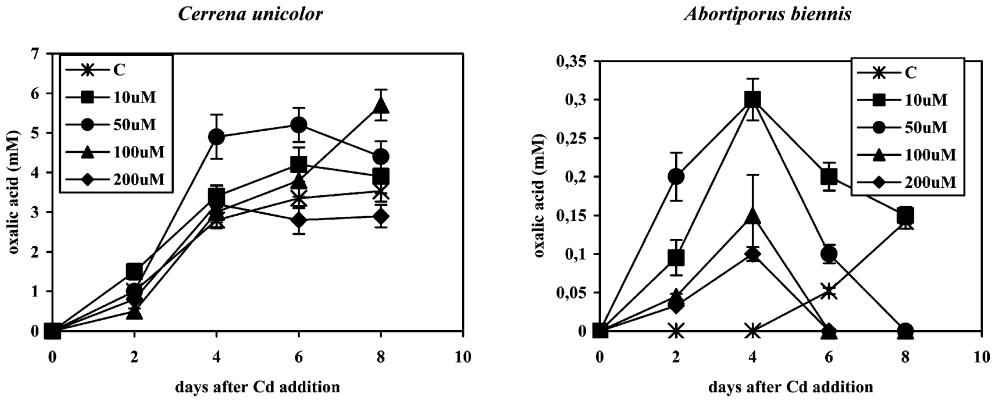

Figure 1. Secretion of oxalic acid in *C. unicolor* and *A. biennis* cultures. Values represent means ± SD ($n = 3$).

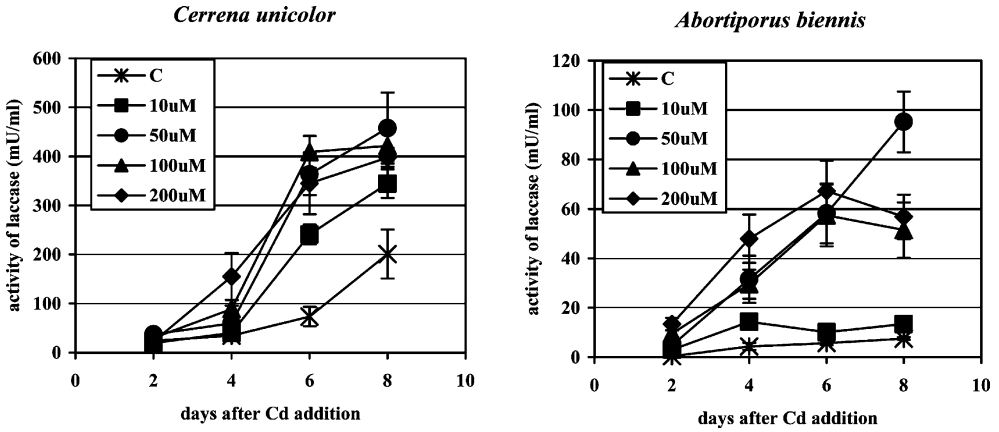

Figure 2. Production of extracellular laccase in *C. unicolor* and *A. biennis* cultures. Values represent means ±SD ($n = 3$).

extracellular laccase activities were higher in *C. unicolor* than in *A. biennis*, irrespective of whether Cd was present or not.

Fungal production of thiol compounds

C. unicolor and *A. biennis* responded to Cd exposure by an increase in concentrations of thiols such as the glutathione precursors Cys (Figure 3) and γ-EC (Figure 4), GSH (Figure 5), and GSSG (Figure 6), depending on the respective Cd concentration and exposure time. Generally, concentrations of thiols were higher in *C. unicolor* than in *A. biennis*, which was also evident for control cultures lacking Cd. As an overall tendency that could be estimated from Figures 3–6, the relative (i.e., in relation to controls) increase in concentrations of the respective thiol compound upon a given amount of Cd was higher in *A. biennis* than in *C. unicolor*. This was especially pronounced upon exposure to 100 and 200 μM Cd found to cause a significantly higher Cd bioaccumulation in *A. biennis* than in *C. unicolor* (Table 2). These differences in thiol compound concentrations indicate species-specific responses to Cd. Neither in *A. biennis* nor in *C. unicolor* PCs could be detected after 8 days of Cd exposure.

Discussion

Fungal growth and distribution of Cd in mycelia

The exposure of fungi to heavy metals is often accompanied by growth inhibition effects and morphological changes of the growing mycelia (Baldrian 2003), as also observed within the present study. *C. unicolor* is obviously less sensitive

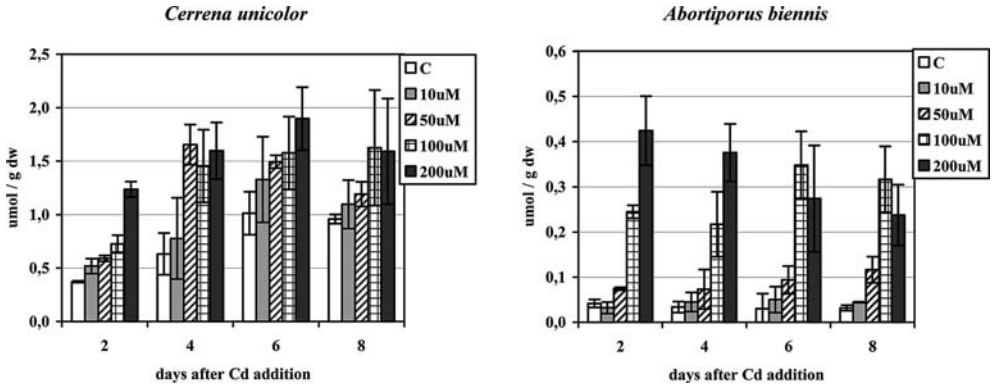

Figure 3. Cysteine content of *C. unicolor* and *A. biennis* during 8 days after Cd addition. Values represent means ± SD ($n = 3$).

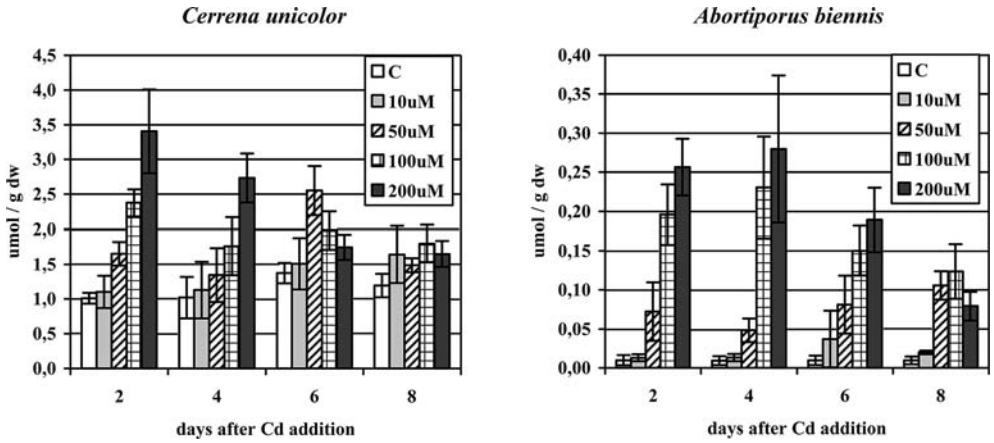

Figure 4. γ-EC content of *C. unicolor* and *A. biennis* during 8 days after Cd addition. Values represent means ± SD ($n = 3$).

towards Cd than *A. biennis* as indicated by its less inhibited growth in presence of Cd (Table 1). This can be explained by lower intracellular amounts of Cd in *C. unicolor*, where bioaccumulated Cd concentrations were significantly lower than in *A. biennis* upon exposure to 100 and 200 μM Cd (Table 2). Cd application resulted in the development of dense aerial hyphae in *C. unicolor* whereas in *A. biennis* no aerial hyphae developed under such conditions, instead the *A. biennis* mycelia became very fragile. Cells of fungal aerial hyphae may secrete small hydrophobic proteins called ''hydrophobins'', which confer hydrophobicity on the fungal surface (Wessels 1997). Genes encoding for hydrophobin precursors were found to be downregulated upon Cd exposure in the ectomycorrhizal fungus *Paxillus involutus* (Jacob *et al.* 2004). This was accompanied by the development of an easily wettable phenotype, similar to the effect of Cd on *A. biennis* mycelia observed in our study. Clearly, such possible effects require more detailed investigations.

The amount of biosorbed Cd significantly exceeded that of bioaccumulated Cd in *C. unicolor*, whereas the biosorbed and bioaccumulated Cd concentrations showed the same order of magnitude in *A. biennis* (Table 2). The surfaces of fungal cells seem to act as ion exchangers. In fungal cell walls, metal cations are bound predominantly to chitin, cellulose, and melanins lacking acidic groups, and this kind of binding to cell walls is probably the most significant biosorption mechanism (Fogarty & Tobin 1996; Vodnik *et al.* 1998). In *Rhizopus arrhizus*, Zn biosorption was predominantly due to Zn binding to wall chitin/chitosan, which are polymers of

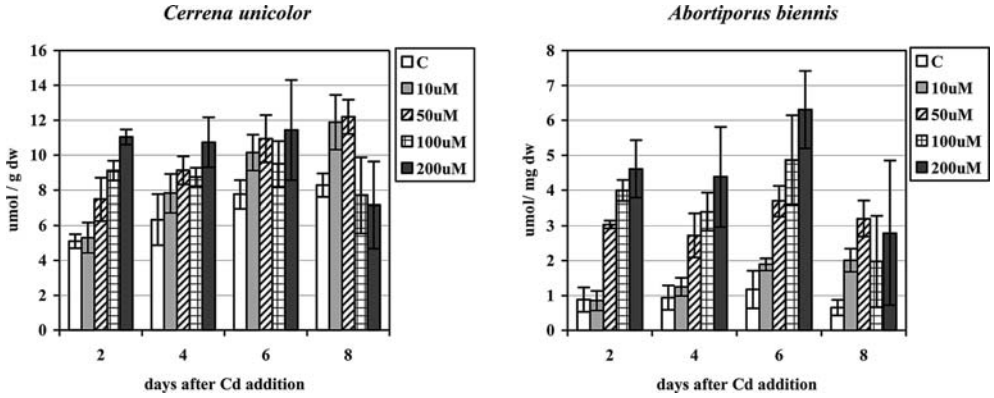

Figure 5. GSH content of *C. unicolor* and *A. biennis* during 8 days after Cd addition. Values represent means ± SD ($n = 3$).

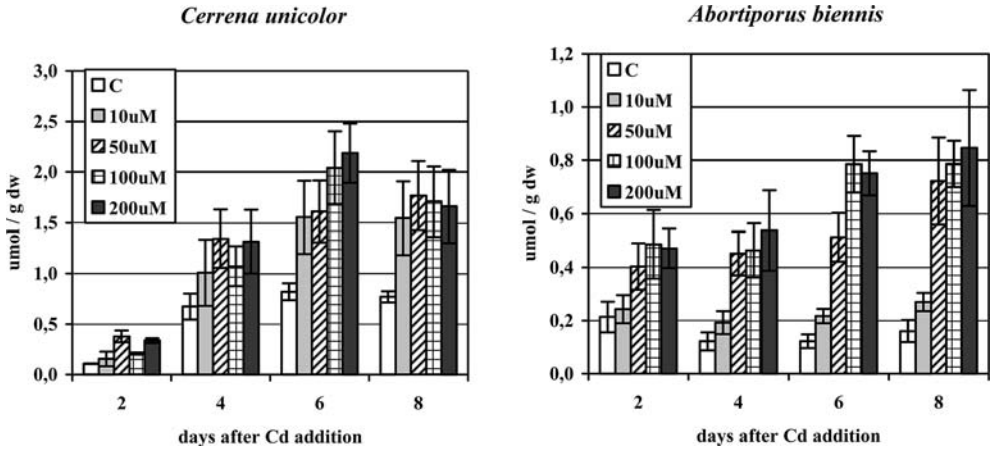

Figure 6 GSSG content of *C. unicolor* and *A. biennis* during 8 days after Cd addition. Values represent means ± SD ($n = 3$).

N-acetyl-D-glucosamine and have a propensity to bind heavy metal ions by chelation (Krantz-Rülcker *et al*. 1995). From the quantitative point of view, surface sorption usually accounts for the larger proportion of total metal fixation but this effect may undergo individual variations, depending on the respective organism and metal. Species-specific variations in the proportions of the biosorbed and bioaccumulated amounts of Cd are also evident from our study (Table 2). Frey *et al*. (2000) have examined the heavy metal localization in hyphae of the ectomycorrhizal fungus *Hebeloma crustuliniforme* and found that extracellular complexation involving metal binding to cell walls was the predominant Cd fixation mechanism, whereas both extracellular complexation and cytosolic sequestration were responsible for Zn fixation under exposure to toxic Zn concentrations.

Fungal secretion of oxalate

In addition to biosorption onto cell wall constituents, microorganisms can prevent damage resulting from excess heavy metal concentrations by immobilization through precipitation in their cell walls or extracellular matrices. Examples for this are the formation of oxalate crystals (Jarosz-Wilkołazka & Gadd, 2003), Cd sulphides (Mehra *et al*. 1994), and Cd fixation by formation of immobile polyphosphate complexes (Joner & Leyval, 2001). Among wood-rotting basidiomycetes, oxalate production is well documented for both white rot and brown rot fungi (Shimada *et al*. 1997; Machuca *et al*. 2001) and was shown, e.g., around hyphae of fungal strains growing on wood treated with copper-based preservatives (Clausen & Green 2003). The production of oxalate provides an organic anions capable of forming a complex with the metal cations, which subsequently may result in the formation of insoluble metal–oxalate complexes. The formation of such complexes may provide a mechanism whereby oxalate-producing fungi can tolerate environments containing high concentrations of toxic metals. Upon Cd exposure, *C. unicolor* produced considerably higher amounts of oxalate (Figure 1) and was clearly less inhibited in its growth (Table 1) than *A. biennis*, which is in support of a Cd-detoxifying function of oxalate in this fungus. Furthermore, oxalate chelates and stabilizes Mn(III), provides H_2O_2, and buffers the environment, which all are important functions for the performance of the ligninolytic system of white rot basidiomycetes (Shimada *et al*. 1997; Schlosser & Höfer, 2002).

Effect of Cd on extracellular laccase activities

Activities of wood-rotting fungi in degradation of the lignin macromolecule and also of other organic compounds depend on the environmental conditions and the respective fungal species. If such organisms are faced with high concentrations of metals in their environment, the production of ligninolytic enzymes can be affected at the levels of transcriptional and translational regulation. In white rot basidiomycetes, laccase is strongly regulated at the gene transcription level by the addition of metals such as Cu and Mn (Palmieri *et al*. 2000; Soden & Dobson 2001; Galhaup *et al*. 2002). Consensus sequences of putative metal responsive elements (MREs), which interact with metal-responsive transcription factors, were identified in promoter regions of laccase genes of white rot fungi (Palmieri *et al*. 2000; Galhaup *et al*. 2002). The organization of such MREs matches those known from promoter regions of metallothionein genes of other eukaryotes (Thiele 1992). It was previously shown that Cd can act as a laccase inducer in *Trametes pubescens* (Galhaup *et al*. 2002), *Phanerochaete chrysosporium*, *Stereum hirsutum* (Baldrian *et al*. 1996), *Pleurotus ostreatus* (Baldrian *et al*. 2000), and *Paxillus involutus* (Jacob *et al*. 2004). Our earlier data demonstrated that application of $CdCl_2$ to 10-day-old cultures of *T. versicolor* significantly enhances extracellular laccase titres (Jarosz-Wilkołazka *et al*. 2002). The extend of increase in laccase activity was dependent on the age of the cultures. In the present study, Cd ions were added to fungal cultures during logarithmic growth. It remains to be elucidated whether the observed increases in laccase activities upon Cd exposure (Figure 2) indicate a direct role of laccases in heavy metal detoxification or rather represent an indirect response due to unspecific or indirect regulatory effects of Cd *via* oxidative stress. In white rot fungi, different ligninolytic enzymes have been shown to be induced by hydrogen peroxide and hydroxyl radicals that also could be expected upon oxidative stress (Li *et al*. 1995; Belinky *et al*. 2003). The higher laccase activities in presence of Cd (Figure 2) and the lower sensitivity towards Cd (Table 1) observed with *C. unicolor*, as

compared to *A. biennis*, may indicate an as yet unknown effect of laccase in protecting *C. unicolor* from toxic Cd concentrations. Cd stress results in laccase induction and production of polyphenolic compounds in *P. involutus*, and melanins formed from phenolic compounds by laccases possess potential metal binding sites (Jacob *et al.* 2004).

Fungal production of thiol compounds

Cd stress obviously results in a complex regulation of the cysteine and glutathione synthesis at the enzymatic and genetic level. GSH possesses a high chemical reactivity and participates in both the elimination of reactive oxygen species *via* thiol-disulfide redox reactive reactions and the binding of heavy metals. It can be hypothesized that under Cd stress a substantial proportion of reduced glutathione (GSH) is removed from the glutathione pool, in order to detoxify the metal by intracellular chelation. Cd-glutathione complexes were the first to appear in *Candida glabrata*, later on also other Cd complexes evolved over the time of metal exposition (Barbas *et al.* 1992). Glutathione- and PC-containing CdS crystallites were found upon exposure of this fungus to Cd (Mehra *et al.* 1994). In *Saccharomyces cerevisiae*, Cd detoxification depends primarily on binding by glutathione in the form of bis(glutathionato)Cd and the sequestration of such complexes into vacuoles by the ABC-transporter YCF1 (Li *et al.* 1997). Under oxidative stress, glutathione may be shifted to its oxidized form (GSSG) by an increasing GSH oxidation and/or decreasing GSSG reduction catalysed by glutathione reductase, thereby altering the GSH/GSSG ratio considered as an indicator for the cellular redox state (Schützendübel & Polle 2002). Cd accumulation induced oxidative stress in *S. cerevisiae* (Brennan & Schiestel 1996). Although Cd is not a transition metal and therefore is not assumed to directly interfere with cellular oxygen metabolism, it may indirectly contribute to oxidative stress due to an initial depletion of GSH and protective enzymes (Schützendübel & Polle 2002). After 2 days of cultivation in presence of 100 and 200 μM Cd, intracellular Cd concentrations in *A. biennis* were approximately 2.5- and 10-fold higher than in *C. unicolor*, respectively (Table 2). At this time, *A. biennis* exhibited GSH/GSSG ratios of about 8 and 10 upon 100 and 200 μM Cd, respectively, whereas the control value was approximately 4 (calculated from Figures 5 and 6). Thus, exposure to 100 as well as 200 μM Cd approximately doubled the respective GSH/GSSG ratio in *A. biennis* which could indicate a role of GSH in initial Cd chelation. In *Mucor racemosus*, exposure to 100 μM Cd for 10 days resulted in a GSH/GSSG ratio of 53 compared to a control value of 33 (Miersch *et al.* 2001). Increased thiol compound levels upon heavy metal exposure were also found in aquatic hyphomycetes (Miersch *et al.* 1997, 2001). No increase in the GSH/GSSG ratio upon Cd addition was observed for *C. unicolor*, where values of about 44 and 33 at 2-day-exposures to 100 and 200 μM Cd, respectively, were lower than the control value of approximately 48 (calculated from Figures 5 and 6).

Although we were not able to detect PCs in *C. unicolor* or *A. biennis*, this does not rule out the possible occurrence of PCs not accessible with the analytical method applied within the present study. In plants, an initial depletion of GSH upon heavy metal stress was often observed, due to an increasing GSH consumption for PC production (Schützendübel & Polle 2002).

The possible contribution of MTs to Cd detoxification in *A. biennis* and *C. unicolor* remains to be investigated. MTs represent cysteine-rich metal binding proteins of low molecular weight (Cobbett & Goldsbrough 2002). Small copper binding cysteine-rich (30%) MTs were described for the ectomycorrhizal basidiomycetes *Laccaria laccata* and *P. involutus* (Howe *et al.* 1997). Recently, 3-kDa molecular mass compounds related to MTs were found after exposition of *P. involutus* to Cd (Courbot *et al.* 2004).

Conclusions

The aim of this study was to provide new insights into mechanisms adopted by wood-rotting fungi to deal with toxic metals, in order to expand our knowledge about their ecology and their potential for biotechnological applications. The observed species-specific responses in thiol compound concentrations, the differences in the GSH/GSSG ratios of the two fungi, as well as the different sensitivities of the two fungi towards Cd may be caused by the remarkable differences in concentrations of bioaccumulated Cd. This implies that

C. unicolor possesses a more efficient system than *A. biennis* to keep intracellular Cd concentrations low. Taken together, the results of the present study suggest two main Cd resistance mechanisms employed by *A. biennis* and *C. unicolor* at varying extends: (i) biosorption of Cd ions onto the fungal cell walls and/or their complexation by extracellular compounds such as oxalate and (ii) intracellular bioaccumulation and complexation by thiol compounds.

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