Cadmium uptake and subcellular compartmentation in the ectomycorrhizal fungus *Paxillus involutus*

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Cadmium uptake and subcellular compartmentation in the ectomycorrhizal fungus *Paxillus involutus* were investigated using radiotracer flux analyses. Concentration-dependent Cd^{2+}-uptake kinetics were characterized by a smooth, non-saturating curve that could be dissected into linear and saturable components. The linear-uptake kinetic component was interpreted as representing binding of Cd to apoplastic components, whereas the remaining saturable component was the result of carrier-mediated transport across the plasma membrane. Cell-wall-bound Cd was almost completely removed during desorption from cell-wall preparations. Cd^{2+} desorption from intact mycelium was found to be a function of time involving three compartments corresponding in series to cell wall (50%), cytoplasm (30%) and vacuole (20%), when mycelia were exposed to a 0·05 µM Cd concentration. At 4 °C, most of the Cd recovered was due to the cell-wall-bound fraction, suggesting that transport across the plasma membrane is a metabolically mediated process. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) inhibited Cd accumulation in *P. involutus* mycelia by up to 28%, which indicates that transport of Cd^{2+} was partially dependent on the membrane potential. Cd^{2+} uptake into symplasm is linked to Ca^{2+} transport, as revealed by the inhibition of Cd accumulation by the Ca^{2+} ionophore A23187. The present work demonstrates the ability of the ectomycorrhizal fungus *P. involutus* to take up and further accumulate Cd in different compartments. Binding of Cd onto cell walls and accumulation of Cd in the vacuolar compartment may be regarded as two essential metal-detoxification mechanisms. These data represent a first step towards the understanding of metal-tolerance mechanisms in mycorrhizal fungi.

Keywords: Cd uptake and compartmentation, Cd desorption, ectomycorrhizal fungus, *Paxillus involutus*

INTRODUCTION

The response of mycorrhizal fungi to toxic metals is of importance in view of their interest in the reclamation of polluted sites and their influence on plant growth and productivity. The decrease of metal phytotoxicity by mycorrhizal fungi has been widely demonstrated (Jones & Hutchinson, 1986; Dixon & Buschena, 1988; Colpaert & Van Assche, 1993). Binding of metals to cell walls, sequestration of metals either by binding proteins or polypeptides, or by complexation in vacuoles could alleviate the toxic effects of free metal ions in cells by effectively lowering their intracellular concentration (Ortiz *et al.*, 1992; Gadd, 1993; Galli *et al.*, 1994; Leyval *et al.*, 1997). However, these tolerance mechanisms are not well understood in mycorrhizal fungi and most data obtained so far concern yeasts and saprotrophic fungi (Ortiz *et al.*, 1992, 1995; Gadd, 1993).

Understanding heavy-metal tolerance very often first requires a knowledge of the basic mechanisms of metal absorption into the cells. Most studies on metal-ion

transport in fungi have concerned K^+ and Ca^{2+}, largely because of their importance in fungal growth, metabolism and differentiation (Gadd, 1993). Most of our knowledge concerning metal-uptake and compartmentation mechanisms is based on studies with yeasts and filamentous fungi other than ectomycorrhizal species. Filamentous fungi live in very different habitats and it was recently highlighted by Burgstaller (1997) that investigations with fungi that represent distinct habitats (mycorrhizal fungi, wood-decomposing fungi and the *Achlya* spp.) are urgently needed. Indeed, mycorrhizal fungi have a unique feature as compared with most of the fungi studied so far in that they are able to form a symbiotic structure with plant roots, providing a unique tool in phytoremediation programmes. Studies on the transport of toxic metal species across fungal membranes could be of interest to engineer ectomycorrhizal fungi with either enhanced or reduced uptake capacities, yielding the possibility of using mycorrhizal trees to phytoremediate polluted soils.

Cadmium is known to be a non-essential element, and can be toxic at very low concentrations. This metal is also ubiquitous in sewage sludges, industrial wastes and mining sites. Uptake of Cd^{2+} across the plasma membrane of root cells has been shown to occur via a concentration-dependent process exhibiting saturable kinetics (Cataldo *et al.*, 1983; Mullins & Sommers, 1986; Costa & Morel, 1993). The saturable nature of Cd uptake in these studies suggests that Cd is taken up via a carrier-mediated system. Conversely, Cd uptake in barley is not under metabolic control but is primarily controlled by diffusion (Cutler & Rains, 1974).

Paxillus involutus, which has become one of the most intensively studied ectomycorrhizal fungi (Chalot *et al.*, 1996), is an abundant species in many forest ecosystems and has been one of the most widely found species on industrial wastes polluted by heavy metals. This fungus also plays important roles in limiting heavy-metal toxicity, and the molecular mechanisms involved in Cd tolerance are currently being studied in our laboratory. In this study, we used the absorption/desorption procedure, which has been widely applied to higher plants (Kochian & Lucas, 1982; Rauser, 1987; Godbold, 1991; Lasat *et al.*, 1998) to characterize ^{109}Cd uptake and subcellular compartmentation in *P. involutus* mycelium.

METHODS

Organism and media. The ectomycorrhizal fungus used was an isolate of *Paxillus involutus* (Batsch) Fr. (ATCC 200175), which was originally isolated from a fruiting body growing under *Betula pendula* (Roth.) on coal waste in Midlothian, Scotland. It was maintained in Petri dishes by successive transfer on cellophane-covered agar medium containing modified Melin–Norkrans (MMN) medium (Chalot *et al.*, 1996).

Cd-uptake experiments. In the standard assay, discs of mycelium were cut from the actively growing edge of 10-d-old colonies using a 15 mm diameter cork borer. The discs were floated for 30 min in a solution (standard assay medium) containing 0·5 mM $CaCl_2$, 2 mM MES and 0·05 μM Cd containing 3·8 nM ^{109}Cd [4·3 mCi mmol^{-1} (15·9 × 10^7 Bq mmol^{-1})] at pH 4·5 and 20 °C. Ca^{2+} was used to structurally stabilize membranes and cell walls (Marschner, 1995). Because the accumulation rate was linear from 3 to 60 min, a 30 min-uptake period was chosen to investigate uptake, whilst minimizing the possibility of ^{109}Cd loss by efflux across the plasma membrane to the external solution. At the end of the incubation period, discs were briefly rinsed in fresh uptake solution from which Cd was omitted, to remove the surface film of radiolabelled solution, dried and weighed before gamma activity was determined. When needed, the pH was adjusted with HCl or NaOH or the temperature adjusted to 4 °C. For the determination of concentration-dependent kinetics, a Cd concentration range of 0–50 μM was used.

Time course of Cd^{2+} desorption from mycelia. For the determination of desorption rates, discs were first exposed for 30 min or 12 h to the standard assay medium, then briefly rinsed in the uptake solution from which Cd was omitted, and finally floated on a desorption solution containing 5 mM $CaCl_2$ in 2 mM MES (pH 4·5) at 4 °C to initiate Cd desorption. The use of a low temperature for Cd desorption prevented the reuptake of this metal, as suggested by Hart *et al.* (1998). At various time intervals, 200 μl aliquots of the desorption solution were taken out and gamma activity was determined. Desorption of ^{109}Cd from mycelia in the external solution was monitored for 10 h.

Mycelium cell-wall preparations. Cell-wall preparations were obtained by immersing discs of intact *P. involutus* mycelia in a methanol/chloroform solution (2:1, v/v) for 3 d. In a preliminary experiment it was found that this treatment gave rise to lipid-free mycelium cell-wall preparations that generally maintained the same shape and size as intact mycelia. Small amounts of proteins were still present on such preparations. Following this treatment, cell-wall preparations were washed in a number of changes of deionized water for 2 d. Discs of either intact or methanol/chloroform-treated mycelia were then incubated in the standard assay medium for various lengths of time (3–60 min) and then either briefly rinsed in the uptake solution from which Cd was omitted (undesorbed), or desorbed at 4 °C in 2 ml desorption solution for 20 min. Subsequently, discs of mycelia or mycelium cell-wall preparations were dried, weighed and ^{109}Cd was quantified by gamma detection.

Effect of metabolic inhibitors on Cd accumulation. Mycelium discs were preincubated for 20 min at 20 °C in a solution containing 0·5 mM $CaCl_2$, 2 mM MES (pH 4·5) in the presence of various metabolic inhibitors [100 μM verapamil, 10 μM A23187, 10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 10 μM dicyclohexylcarbodiimide (DCCD) or 1 μM nonactin], and then incubated in the standard assay medium, supplemented with the inhibitor. Discs of mycelia were subsequently rinsed in the uptake solution from which Cd was omitted and floated at 4 °C in a desorption solution containing 5 mM $CaCl_2$ in 2 mM MES (pH 4·5). At various time intervals, 200 μl aliquots of the desorption solution were taken out and the gamma activity was determined. Desorption of ^{109}Cd from mycelia in the external solution was monitored for 7 h. Metabolic inhibitors were prepared in 95 % ethanol. Control treatments consisting of the same concentration of ethanol but without inhibitors were included.

Because Cd and other cations may adhere to glass surfaces, plastic material was used for all preincubation, uptake and

desorption solutions. Labelled cadmium (^{109}Cd) was purchased from Amersham Laboratories. Other chemicals were from Sigma.
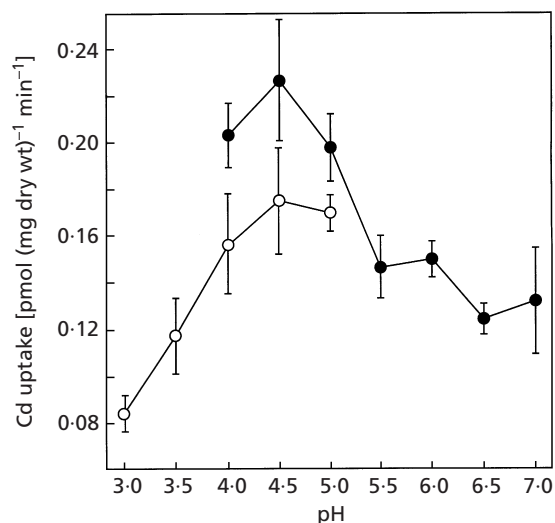
RESULTS

Time and pH dependences

In the standard conditions, the pH of the incubation medium had a strong effect on the uptake of Cd (Fig. 1). Of the pH values examined, pH 4·5 was found to be optimal for uptake of Cd whereas it decreased when the pH was raised to 7·0. The uptake of Cd^{2+} by *P. involutus* mycelium was specifically enhanced by a MES buffer system, as compared with a citrate buffer system (Fig. 1). To avoid possible complexation of Cd^{2+} by citrate, pH 4·5 was maintained in further experiments with MES buffer, which is known to have minimal metal-complexing ability. Time-dependent Cd accumulation in mycelia of *P. involutus* was linear for at least 60 min (Fig. 2b). Thereafter, the rate of accumulation decreased until it reached a plateau phase after 8 h (Fig. 2a).

Influence of Cd concentration

Cd uptake within a 0–50 µM concentration range was characterized by a smooth, non-saturating curve (Fig. 3b). Uptake kinetic isotherms could be readily dissected into linear and saturable components using an Eadie–Hofstee plot (v against v/S) which allowed determination of the Michaelis constant ($K_m = 32$ nM) and
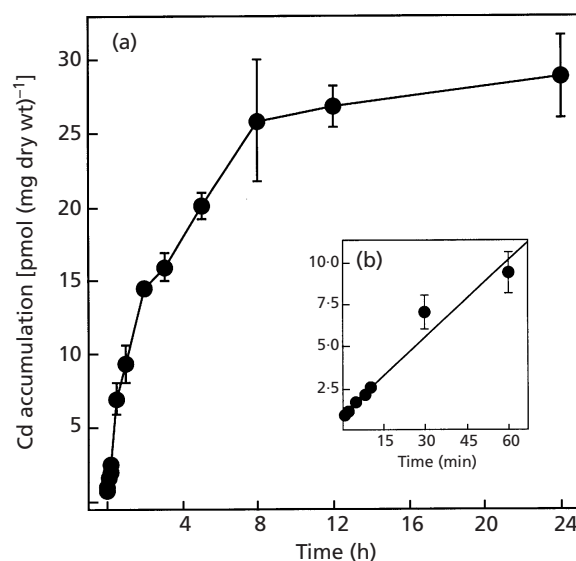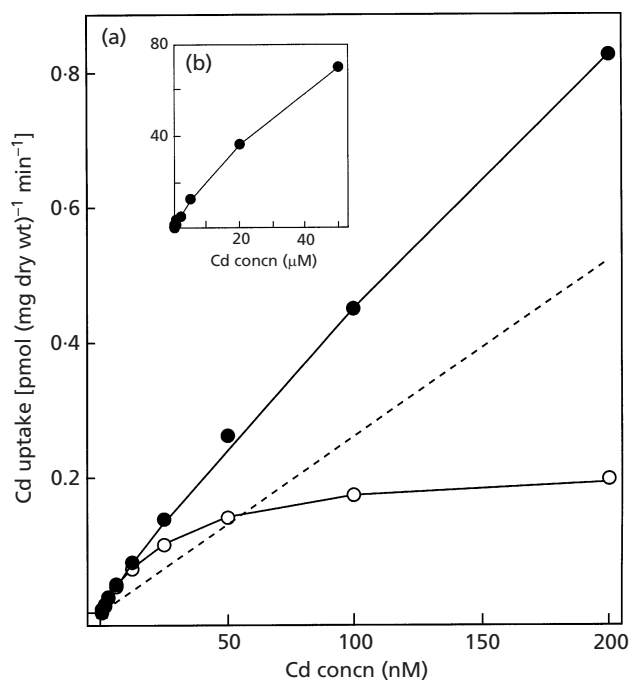
Fig. 2. Time course of Cd accumulation in mycelia of *P. involutus*. Mycelium discs were exposed for long (a) or short (b) incubation periods to an uptake solution containing 0·05 µM Cd labelled with ^{109}Cd (4·3 mCi mmol^{-1}), 0·5 mM CaCl$_2$ in 2 mM MES at pH 4·5. Discs were rinsed in uptake solution in which Cd was omitted, dried and weighed prior to gamma activity determination. The legend on the *y* axis also applies to the inset graph. Data points and error bars represent means ($n = 3$) and SE, respectively. Error bars do not extend outside some symbols.

maximum velocity [$V_{max} = 0·19$ pmol (mg dry wt)$^{-1}$ min^{-1}] for the saturable component obtained in the low-concentration range (Fig. 3a).
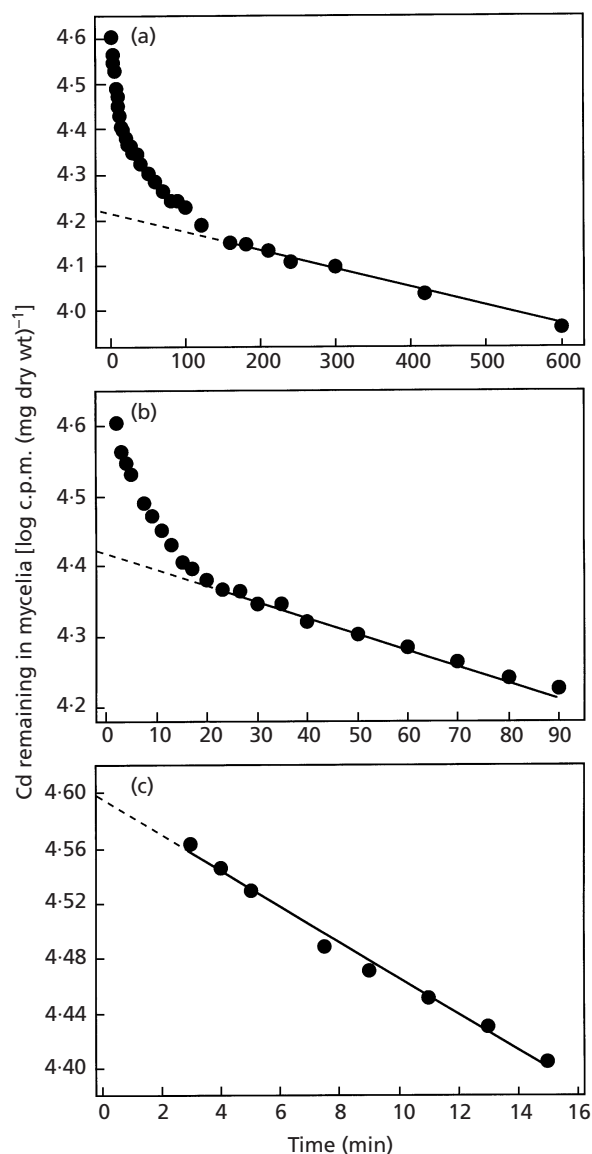
Compartmentation studies

To investigate the subcellular compartmentation of Cd in fungal hyphae, both short-term (30 min) and long-term (12 h) studies were carried out on the time-dependent kinetics of ^{109}Cd desorption from *P. involutus* mycelium. Plots representing a first-order kinetic transformation of Cd desorption (log of Cd remaining in the mycelium as a function of time) could be dissected into three compartments in series, the vacuole, cytoplasm and cell wall (Fig. 4), as previously found for Cd^{2+} (Rauser, 1987) and other ions (Macklon *et al.*, 1996; Lasat *et al.*, 1998). The data agreed well with a system obeying first-order kinetics for efflux from a three-compartment model and the verification of the model was performed by applying kinetic analyses to the experimental data. As recommended by Kochian & Lucas (1982), plots of log content versus time and log efflux versus time yielded curves fitted by the same number of compartments, with identical rate constants for the corresponding compartments from each plot. The straight line drawn through data representing the slowest-exchanging phase (160–600 min) was interpreted to represent Cd efflux from the vacuole (Fig. 4a).

Fig. 1. Effect of external pH on Cd uptake into mycelia of *P. involutus*. Mycelium discs were exposed for 30 min to an uptake solution containing 0·05 µM Cd labelled with ^{109}Cd (4·3 mCi mmol^{-1}), 0·5 mM CaCl$_2$ in 2 mM MES (●) or citrate (○) buffer at the pH values shown. Discs were rinsed in uptake solution in which Cd was omitted, dried and weighed prior to gamma activity determination. Data points and error bars represent means ($n = 3$) and SE, respectively.

Fig. 3. Concentration-dependent kinetics of Cd uptake into mycelia of *P. involutus*. Mycelium discs were exposed for 30 min to an uptake solution containing 0–50 μM Cd (b) labelled with [109]Cd (4·3 mCi mmol^{−1}), 0·5 mM CaCl$_2$ in 2 mM MES at pH 4·5. Details of the low-concentration range, including resolution of overall kinetic curves (●) into saturable (○) and linear (dotted line) components, are given in (a). Discs were rinsed in uptake solution in which Cd was omitted, dried and weighed prior to gamma activity determination. The legend on the *y* axis also applies to the inset graph. The curve represents a typical experiment.

Fig. 4. Short-term desorption of Cd from mycelium of *P. involutus*. After 30 min incubation in an uptake solution containing 0·05 μM Cd labelled with [109]Cd (4·3 mCi mmol^{−1}), 0·5 mM CaCl$_2$ in 2 mM MES (pH 4·5), mycelium discs were briefly rinsed in uptake solution in which Cd was omitted and, to initiate Cd desorption, the discs of mycelium were floated at 4 °C in a desorption solution containing 5 mM CaCl$_2$ in 2 mM MES (pH 4·5). [109]Cd desorption from cell wall, cytoplasm and vacuole into the external solution was subsequently monitored for 10 h (a). The linear component in (a) was subtracted from the data points in (a) to obtain cell wall and cytoplasm desorption (b). Similarly, cell-wall desorption (c) was derived from the curve in (b). Lines represent regressions of the linear portion of each curve extrapolated to the *y* axis. The curves represent a typical experiment.

From the slope of this line we estimated the half-time for Cd efflux from the vacuole (Table 1). The *y* axis intercept of this line was used to calculate the distribution of Cd in fungal cells at the termination of the radioisotope-loading period (Table 1). Subtraction of this linear component from total desorption data (Fig. 4a) yielded a second curve, which was analysed similarly and the straight line drawn was interpreted as representing Cd efflux from the cytoplasm (20–90 min) (Fig. 4b). Efflux from the cell wall (2–15 min) (Fig. 4c) was obtained similarly after subtracting the linear phase associated with the cytoplasmic efflux from the data points plotted in Fig. 4b.

As shown in Table 1, after a 30 min incubation of mycelia in Cd-uptake solution, the cell wall compartment represented 48 % of the total Cd accumulated, whereas the cytoplasm and vacuole represented 32 and 20 % of the total Cd accumulated, respectively. None of the three cellular compartments was saturated by Cd after 30 min incubation, as suggested by a 12 h exposure to Cd (Table 1). Amounts of Cd accumulated were 6, 9 and 13 times higher in cell wall, cytoplasm and vacuole compartments, respectively, than those observed in the

same compartments after a shor-term exposure (Table 1). However, this long-term exposure (12 h) of mycelia to Cd modified the distribution of this ion when compared with the short-term exposure (30 min). Cd

Table 1. Intracellular Cd compartmentation and half-time ($t_{1/2}$) for Cd desorption from different fungal compartments in *P. involutus* mycelia

Incubation time	Cell wall	Cytoplasm	Vacuole
30 min			
Cd (c.p.m.)*	39 646	26 306	16 552
Cd (%)	48	32	20
$t_{1/2}$ (min)†	23	144	729
12 h			
Cd (c.p.m.)*	237 465	228 636	213 820
Cd (%)	35	33·5	31·5
$t_{1/2}$ (min)†	228	809	2280

*c.p.m. values were obtained from the intersection of the extrapolated linear components shown in Fig. 4 with the *y* axis.

†$t_{1/2}$ values were calculated from the slope of the linear components shown in Fig. 4.

was nearly equally distributed among the three cellular compartments (cell wall, cytoplasm and vacuole). After the short-term Cd exposure, half-times ($t_{1/2}$) for Cd desorption were 23, 144 and 729 min for cell wall, cytoplasm and vacuole, respectively. These half-times were higher when the desorption was performed after a long-term exposure.

Cd accumulation by cell-wall preparations

The efficacy of the desorption regimen at removing cell-wall Cd was further tested by investigating the time course of Cd accumulation (Fig. 5) in living mycelia and cell-wall extracts prepared as described in Methods. In intact, undesorbed mycelia, Cd accumulation was linear with time (Fig. 5a). In desorbed intact mycelia, Cd accumulation was also linear, but the slope of this line was lower than that of the undesorbed intact mycelia. Cd accumulation in undesorbed cell-wall preparations was linear (Fig. 5b) and exceeded Cd accumulation in undesorbed intact mycelia (Fig. 5a). After a 20 min desorption treatment, most of the accumulated Cd was removed (Fig. 5b). Thus, it appears that most of the desorbed Cd in intact mycelia was due to the loss by cell walls. Consequently, in subsequent experiments using metabolic inhibitors, a 20 min desorption regimen was used following radioactive uptake to remove most of the mycelium cell-wall Cd (cell-wall-bound Cd) and to quantify Cd transport into the intracellular compartments (intracellular Cd).

Effect of low temperature

After 30 min incubation with 0·05 µM Cd, a low-temperature treatment (4 °C) decreased total accumulation of [109]Cd by 59 % compared with the control (Table 2). A detailed examination of Cd distribution under these conditions showed that the cell-wall compartment retained 75 % of the radioactivity whereas
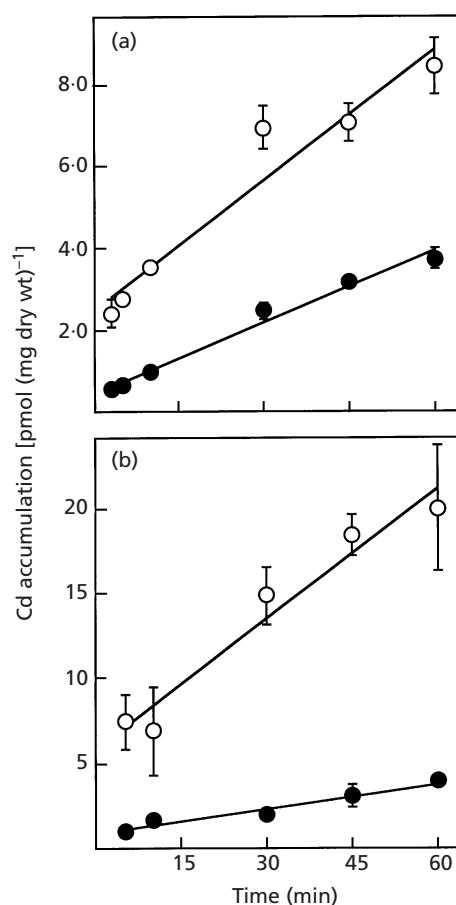
Fig. 5. Effect of desorption on Cd accumulation in intact (a) and methanol/chloroform-treated (b) *P. involutus* mycelia. Discs of intact or methanol/chloroform-treated mycelia were exposed to the uptake solution containing 0·05 µM Cd labelled with [109]Cd (4·3 mCi mmol^{-1}), 0·5 mM CaCl$_2$ in 2 mM MES (pH 4·5). At the end of the incubation periods shown, either discs were rinsed (○) in uptake solution in which Cd was omitted, dried, weighed and the gamma activity counted, or they were desorbed (●) for 20 min at 4 °C in 2 mM MES (pH 4·5), 5 mM CaCl$_2$ before being dried, weighed, and their gamma activity counted. Data points and error bars represent means (*n* = 3) and SE, respectively. Error bars do not extend outside some symbols.

[109]Cd accumulation was strongly decreased in the cytoplasm and vacuole compartments (Fig. 6, Table 2).

Effect of metabolic inhibitors

Total Cd accumulation was inhibited by 28 and 38 % by the protonophore CCCP and the Ca^{2+} ionophore A23187, respectively (Fig. 6, Table 2). The lower Cd accumulation under A23187 and CCCP treatments was due to a reduced accumulation of [109]Cd in cytoplasm and vacuole (Fig. 6, Table 2). The Ca^{2+} channel blocker verapamil had only a slight effect on Cd accumulation. The H$^+$/ATPase inhibitor DCCD and the K$^+$ ionophore nonactin had no effect on Cd accumulation and compartmentation (Table 2).

Table 2. Effect of low temperature and metabolic inhibitors on Cd accumulation in *P. involutus* mycelia

Cd accumulation values are expressed as c.p.m. (mg dry wt)$^{-1}$. These values were obtained from the data of Fig. 6 and after extrapolation to the y axis of the linear components representing the three cellular compartments in series (vacuole, cytoplasm, cell wall) (for detailed explanations, see Results). Data are expressed as means $\pm$ SE of three replicates. The proportion of Cd of each cellular compartment is shown in parentheses. Asterisks indicate significant differences from the control treatment (ANOVA, $P < 0.001$).

Treatments (used for loading of ^{109}Cd)	$10^{-3} \times$ Cd compartmentation		
	Cell wall	Cytoplasm	Vacuole
Control	$39 \cdot 2 \pm 1 \cdot 7$ (45·3)	$27 \cdot 1 \pm 2 \cdot 1$ (31·3)	$20 \cdot 3 \pm 1 \cdot 5$ (23·4)
4 °C incubation	$28 \cdot 2 \pm 3 \cdot 1^*$ (75·2)	$7 \cdot 2 \pm 0 \cdot 5^*$ (19·2)	$2 \cdot 1 \pm 0 \cdot 8^*$ (5·6)
Verapamil (100 µM)	$34 \cdot 2 \pm 1 \cdot 9^*$ (42·0)	$26 \cdot 2 \pm 2 \cdot 8$ (32·2)	$21 \cdot 0 \pm 2 \cdot 4$ (25·8)
A23187 (10 µM)	$28 \cdot 7 \pm 2 \cdot 1^*$ (52·4)	$15 \cdot 7 \pm 2 \cdot 3^*$ (28·6)	$10 \cdot 4 \pm 0 \cdot 8^*$ (19·0)
CCCP (10 µM)	$34 \cdot 3 \pm 3 \cdot 2^*$ (53·1)	$19 \cdot 4 \pm 1 \cdot 9^*$ (30·0)	$10 \cdot 9 \pm 1 \cdot 4^*$ (16·9)
DCCD (10 µM)	$38 \cdot 9 \pm 1 \cdot 1$ (45·2)	$27 \cdot 3 \pm 3 \cdot 3$ (31·7)	$19 \cdot 9 \pm 2 \cdot 0$ (23·1)
Nonactin (1 µM)	$40 \cdot 2 \pm 2 \cdot 4$ (45·4)	$28 \cdot 1 \pm 2 \cdot 0$ (31·8)	$20 \cdot 2 \pm 2 \cdot 3$ (22·8)

DISCUSSION

In the present study, we have performed, to our knowledge, the first physiological characterization of heavy-metal uptake and compartmentation in an ectomycorrhizal fungus. The application of radiotracer desorption analysis only allows for a semi-quantitative but indirect estimate of ion fluxes and loading in the vacuole, cytoplasm and cell wall. However, this technique has been used to provide valuable information on the efflux of several ions such as K$^+$ (Kochian & Lucas, 1982), Cu^{2+} (Thornton, 1991), Zn^{2+} (Lasat et al., 1998), Co^{2+} (Macklon & Sim, 1987) and Cd^{2+} (Rauser, 1987; Godbold, 1991). Mechanisms for accumulation of metals in the cells probably involve transport regulation at several different sites within the mycelium, including binding to the cell walls, uptake across the plasma membrane and uptake across the tonoplast.

Kinetics of uptake

Linear, time-dependent accumulation of Cd in roots has been reported previously in experiments using low Cd concentrations (20–500 nM) (Cataldo et al., 1983; Hardiman & Jacoby, 1984; Homma & Hirata, 1984), whereas saturable, time-dependent Cd accumulation was reported in barley (Cutler & Rains, 1974). An increase of pH in the incubation medium led to an inhibition of Cd uptake. At high pH values, Cd^{2+} ions may be hydrolysed and possibly precipitate as sparingly soluble hydroxides (Kwan & Smith, 1991). These lower Cd concentrations also induced a reduced uptake by fungal cells. Previous studies with radish and soybean showed that Cd uptake increased with decreasing soil pH (Lagerwerff, 1971; Miller et al., 1976).

The K_m value of 32 nM for Cd uptake by fungal cells is consistent with those reported for various herbaceous species such as wheat (Hart et al., 1998), lupin (Costa & Morel, 1993) and soybean (Cataldo et al., 1983). The value found for maximum velocity (V_{max}) is lower than those of Hart et al. (1998). As discussed by these authors, the linear-uptake kinetic component can be interpreted as representing binding of Cd to apoplastic components, whereas the remaining saturable component is the result of carrier-mediated transport across the plasma membrane.

Cell-wall binding

The confounding effect of cell-wall binding can be eliminated to some degree by desorbing cell-wall-bound Cd from mycelia following radioactive treatment. Binding of Cd to the cell walls may represent a substantial fraction of the metal accumulated by mycelia and may also be part of the mechanisms by which mycorrhizal fungi tolerate high amounts of metals. Methanol/chloroform-treated mycelia further showed that cell walls may bind high amounts of Cd and that this fraction was removed very quickly with the desorption regimen used. However, this treatment also induced a higher recovery of Cd in cell-wall preparations compared with intact mycelia. In agreement with Hart et al.
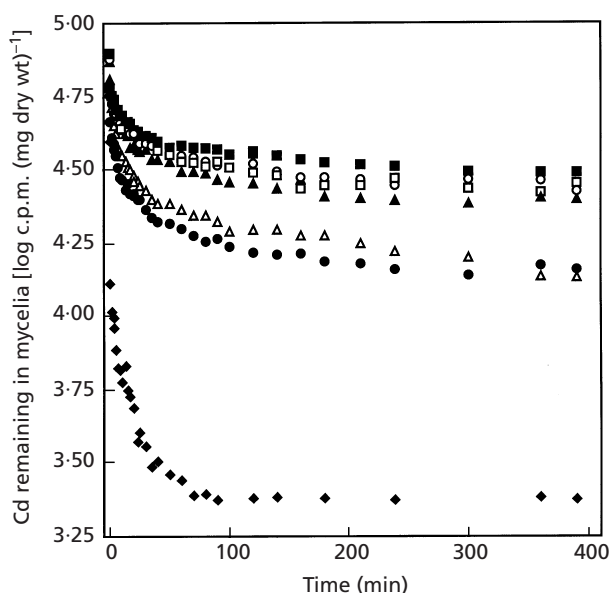
Fig. 6. Effect of metabolic inhibitors and low temperature on Cd compartmentation in *P. involutus* mycelia. Fungal discs were preincubated for 20 min in a solution containing 0·5 mM CaCl₂, 2 mM MES (pH 4·5) in the absence (■) or in presence of various metabolic inhibitors [100 µM verapamil (▲), 10 µM A23187 (●), 10 µM CCCP (△), 10 µM DCCD (○) or 1 µM nonactin (□)], and then incubated for 30 min in the standard assay medium, supplemented with the inhibitor. Discs were rinsed and then desorbed at 4 °C in 2 mM MES (pH 4·5), 5 mM CaCl₂. For the low-temperature treatment (◆), both the uptake and desorption periods were performed at 4 °C. At different time intervals, radioactivity was determined in aliquots of the desorption solution. At the end of the desorption experiment, discs were rinsed, dried, weighed and their gamma activity counted. Each curve represents a typical experiment.

(1998), we found residual proteins that may have contributed to higher levels of metal binding. Ting & Teo (1994) showed that formaldehyde-treated yeast cells consistently showed significantly greater accumulation of Cd than did non-treated cells. It has been proved that chemical modification of the isolated cell walls modifies their capacity to accumulate Cu²⁺ cations. The blocking of amino, carboxyl or hydroxyl groups reduced the amount of Cu²⁺ accumulated, indicating that they may play a role in the binding of Cu²⁺. This in turn indicates that both the protein and the carbohydrate fractions of the cell walls are involved in metal binding (Brady & Duncan, 1994). Cd was found to be bound to negatively charged sites associated with the cell-wall components such as chitin, cellulose, cellulose derivatives and melanins (Galli *et al.*, 1994). Turnau *et al.* (1994) found that Cd could be bound to the outer pigmented layer of the cell wall. It has been previously suggested that tolerance to metal is associated with the formation of pigments and that the activity of tyrosinase, the melanin biosynthetic complex, is stimulated by metals in ectomycorrhizal fungi, probably to increase metal sequestration onto cell-wall pigments (Gruhn & Miller, 1991). This is in good agreement with

recent experiments performed in our laboratory, in which a cDNA encoding a tyrosinase was found to be differentially expressed in *P. involutus* mycelia exposed to Cd (C. Jacob, A. Brun, B. Botton & M. Chalot, unpublished results).

Intracellular uptake

A low-temperature treatment decreased total accumulation of ¹⁰⁹Cd and its distribution in the different compartments, suggesting that transport across membranes is a metabolically mediated process. In filamentous fungi, yeasts and plants, energy-dependent transport of many divalent cations has been demonstrated (Fuhrmann & Rothstein, 1968; Norris & Kelly, 1977; Cataldo *et al.*, 1983; Godbold, 1991; Hart *et al.*, 1998). Divalent cation uptake may be energized by the H⁺ gradient, as found for Co²⁺ and Ni²⁺ uptake in yeast cytoplasm (Okorokov, 1985). CCCP inhibited Cd uptake in *P. involutus* mycelia by up to 28 %, which is in agreement with previous studies showing that transport of divalent cations was dependent on the membrane potential in yeast cells (Borst-Pauwels, 1981; White & Gadd, 1987) and that uptake was strongly inhibited by protonophoric uncouplers that depolarize the cell membrane (Gadd & White, 1989; Tripathi *et al.*, 1995). Since CCCP (a protonophore) partially inhibits Cd uptake, H⁺ may be considered as a counter ion. However, the effect of CCCP is only partial, suggesting that other Cd-uptake mechanisms may play a role. The effect of CCCP may be partially hidden by the higher proportion of cell-wall-bound Cd.

From the present experimental results, the lack of inhibition by nonactin, a K⁺ ionophore, of Cd accumulation provides evidence that the transport system for Cd in *P. involutus* mycelia is not dependent on the K⁺ gradient. The lack of effect of DCCD on Cd uptake by *P. involutus* rules out the involvement of the H⁺/ATPase for H⁺ efflux during Cd uptake. The possibility of Cd uptake via calcium carriers was also tested in *P. involutus*. A23187, a Ca²⁺ ionophore, decreased Cd accumulation, indicating that Ca²⁺ carriers could play a role in Cd²⁺ transport across membranes. Non-essential heavy metals such as Cd are also most likely taken up via plant nutrient transporters or channels that are not completely selective (Clemens *et al.*, 1998). It has been shown that the plant cDNA *LCT1* mediates the uptake of both calcium and cadmium in yeast (Clemens *et al.*, 1998). The study on the interactions between Cd and Ca uptake will be further investigated in our laboratory. After being transported to the cytoplasm, metals may be bound to intracellular substances, the chemical nature of which is not clear. Animals and some fungi, such as *Saccharomyces cerevisiae* and *Neurospora crassa*, induce metallothionein synthesis, whereas plants and some other fungi, such as *Candida glabrata* and *Schizosaccharomyces pombe*, synthesize phytochelatins (small peptides that are not products of RNA translation) in order to complex cytoplasmic Cd (Ortiz *et al.*, 1992; Rauser, 1995). The low-molecular-mass phytochelatin/

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Cd^{2+}/S^{2-} complexes would function as scavengers and carriers of cytoplasmic Cd (Ortiz *et al.*, 1992).

Using compartmentation experiments, we have found significant transport of Cd into the vacuoles. The accumulation of metals in vacuolar phosphate-rich material has been previously suggested in the ectomycorrhizal fungus *Pisolithus arrhizus* (Turnau *et al.*, 1994) and the high nitrogen and sulphur concentrations associated with polyphosphate granules may indicate the occurrence of metal–thiolate binding by metallothionen-like peptides (Galli *et al.*, 1994). Movement of Cd across the tonoplast of the fission yeast cells has been described as occurring by a phytochelatin/Cd transporter. Mutants lacking the ability to accumulate phytochelatin/Cd^{2+} complexes in the vacuole are Cd sensitive (Ortiz *et al.*, 1992). Whatever the mechanism of tonoplast Cd transport, vacuolar compartmentation of Cd would tend to limit symplastic movement of the metal.

Concluding remarks

Metal-uptake and compartmentation studies with symbiotic micro-organisms such as ectomycorrhizal fungi may be of particular importance for phytoremediation strategies. As suggested by Galli *et al.* (1994), ectomycorrhizal symbiosis can play a crucial role in protecting plant roots from heavy metals. The present work demonstrates the ability of the ectomycorrhizal fungus *P. involutus* to take up and further accumulate Cd in different compartments. Binding of Cd onto cell walls and accumulation of Cd in the vacuolar compartment may be regarded as two essential detoxification mechanisms. The present investigation was performed with *P. involutus* cultivated separately from its host plant; therefore it remains to be established to what extent these uptake processes apply to the symbiotic relationship. These data represent a first step towards the understanding of the mechanisms underlying metal tolerance in both ectomycorrhizal fungi and ectomycorrhizal plants.

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