Journal of Industrial Microbiology (1995) 14, 233–239

Metal ion binding by *Pithomyces chartarum* conidia

F. J. Stutzenberger

Ministry of Agriculture and Fisheries, Ruakura Agricultural Research Centre, Hamilton, New Zealand

(Received 4 April 1994; accepted 18 August 1994)

Key words: Copper; Fungus; Iron; Manganese; Metal binding; *Pithomyces*; Zinc

SUMMARY

The binding of metals (Cu, Fe, Mn and Zn) commonly found in soil and decomposing plant material was studied in the saprophytic fungus, *Pithomyces chartarum*. Binding of metallic divalent cations was pH-dependent and temperature-independent; equilibrium occurred within 10 min in stirred suspensions of conidia, but mycelia had no detectable affinity for the metals. Germ tube emergence and elongation were stimulated by high concentrations of Mn^{++} and Zn^{++}, but not by Cu^{++} or Fe^{++}. Metal binding did not obey a simple adsorption isotherm; Scatchard plot analysis indicated two classes of binding sites on the conidial surfaces, one class having association constants about 35-fold greater than those of the other. Calculations based on the conidial surface area as a smooth ellipsoid and the radii of the divalent cations indicated a multilayered coverage of the conidia by the metals at saturation concentrations. Binding sites were stable to boiling, dilute acid and base and lipid solvent extraction. The metals competed with the fungicide, thiabendazole, for binding sites on conidial surfaces.

INTRODUCTION

Fungal cell walls are negatively charged, multilayered matrices largely composed of α- and β-glycans, heteroglycans, chitin, lipid and protein [2,22]. The charged carboxyl, ester sulfate and phosphate groups of cell wall components, enable fungi to bind a variety of cations on their surfaces [reviewed in 19]. This ability is currently receiving widespread attention for its biotechnological application in toxic metal removal from wastestreams, for radionuclide recovery, and in extraction of precious metals from low-grade ores [5,9,13,14,29]. The following study presents a quantitative assessment of the metal binding capacity of conidia and mycelia from the saprophytic fungus, *Pithomyces chartarum*, which produces an unusual, spiculate conidial surface [4]. Four micronutrient metals (Cu, Fe, Mn and Zn) found in high water-soluble concentrations in mixtures of soil and decomposing plant material [reviewed in 1] were tested for their ability to bind to conidia, to stimulate their germination and to inhibit binding of thiabendazole, a systemic fungicide used to control the growth of *P. chartarum* and other soilborne fungi [18,27].

MATERIALS AND METHODS

Organism

Pithomyces chartarum (Berk & Curt) M. B. Ellis strain C conidia were cultured, harvested, washed and dried as previously described [35] to yield approximately 3.3×10^9 conidia per g dry wt.

Correspondence to F.J. Stutzenberger at his present address: Department of Microbiology, Clemson University, Clemson, SC 29634-1909, USA.

Germination medium

The medium (1% glucose, 0.2% Difco (Detroit, MI, USA) vitamin-free casamino acids, and 0.1 M phosphate, pH 6) was prepared in glass-distilled water. Trace element analysis of this medium yielded the following concentrations: Fe, 0.3 μM, Zn, 0.6 μM. Mn and Cu concentrations were below limits of detection. This medium was employed at 26 °C in both a liquid form and solidified with Oxoid IonAgar (Unipath Ltd, Hampshire, UK) (1%) as described by Stutzenberger and Parle [36].

Metal binding studies

The four metals, Cu, Fe, Mn, and Zn, were prepared in 10 mM stock solutions as sulfates of their divalent states. The $FeSO_4$ solution was freshly prepared for each experiment to avoid oxidation to the trivalent state. Routine incubation mixtures contained 5 mg (dry wt) of conidia, 1.0 ml of 0.5 M buffer (acetate in the pH range of 4–6, cacodylate for pH 4.5–7.5, phosphate for pH 6–8 and Tris for pH 7–9), various volumes of metal stock solutions and water to 10 ml. Control mixtures contained buffer and metals without conidia. All mixtures were stirred for 10 min at room temp, filtered (Millipore, 0.45-μm pore size) and assayed for residual metal concentrations. After filtration, differences in free metal concentrations between conidia suspensions and controls were recorded as the amounts bound. Data shown in all figures and tables (except where noted otherwise) are averages of results from independent experiments using two separately cultured and prepared batches of conidia. The standard deviation in metal binding values, based on one set of six replicates for each metal, was $\pm$ 0.052.

Metal ion assay

A Varian-Techtron (Palo Alto, CA, USA) AA5 atomic absorption spectrometer was used for quantitation of metal

concentrations. The wavelengths at which the Cu, Fe, Mn, and Zn analyses were performed in an air/C_2H_2 flame were 324.7, 248.3, 279.5, and 213.9 nm respectively. The practical lower limits for detection were 1 μg L^{-1} for Cu and Mn, 3 μg L^{-1} for Fe and 0.8 μg L^{-1} for Zn. All samples were diluted to appropriate concentrations in 0.5 M HCl and read against standard curves of similar acidified known element concentrations. The average standard deviation of the metal assay (based on six replicate determinations of diluted stock solutions) was ± 0.027.

Thiabendazole binding

Reaction of thiabendazole with *P. chartarum* conidia was done in stirred suspensions at pH 5.0, room temperature, for 10 min as previously described [35]. After removal of conidia by filtration, residual thiabendazole concentrations were determined spectrophotometrically at 298 nm by comparison to similarly treated control solutions receiving no conidia. The amounts of bound thiabendazole were calculated as the decrease in free concentrations after conidia removal and recorded as μg mg^{-1} dry wt.

Metal binding site analysis

The method of Scatchard [32], originally used to study the binding of ions to proteins, was used to define differences between metal binding sites on the conidia surfaces:

$$v = \Sigma_i\, v_i = \Sigma\, i\, \frac{n_i K_i A}{1 + K_i A}$$

where v_i is the average molar ratio of bound ions for the i^{th} binding site on the adsorbing surface, n_i is the number of sites in the i^{th} class of site, K_i is the association constant for the interaction of this class of sites with the ions being bound, and A is the equilibrium concentration of unbound ions in moles per liter. The form of the equation used in graphical analysis was:

$$v/A = K(n - v)$$

A plot of v/A versus v is linear if K is constant. The intercept on the v/A axis is equal to Kn, the slope equals $-K$, and the intercept on the v axis equals n. If binding involves two or more classes of sites, the plot has one or more inflections. Association constants were calculated by the method of Feldman [12].

Degradative enzymes

The enzymes used to test the stability of adsorptive sites on conidia surfaces were purchased from Sigma Chemical Co (St Louis, MO, USA). Each enzyme activity was adjusted to approximately one International Union of Biochemistry unit and used at room temperatuure with pH values as follows: chitinase, 6.0; protease, 7.4; beta-glucuronidase, 4.9; cellulase, 4.6; and acid phosphatase, 4.8.

RESULTS

The initiation of conidia germination in *Pithomyces* (as measured by germ-tube emergence) was stimulated by relatively high concentrations of Mn and Zn. After 2 h of incubation at 26 °C in the liquid casamino acids medium, the percentage of conidia having visible germ-tubes was 39% and 41% in the presence of 0.75 mM Mn or Zn respectively compared to 11% for controls without additional metal. Germ-tube elongation, measured after 10 h incubation on the casamino acids agar medium, was stimulated by Zn (Fig. 1). A comparable degree of stimulation (average of 49% longer) was observed in the presence of 0.75 mM Mn (detailed data not shown), but no stimulation of germ-tube elongation could be detected with 0.1–1.0 mM concentrations of Cu or Fe. This stimulation was apparently not due to contaminating trace elements added with the high concentrations of the element being tested; a combination of Zn, Cu, Mn, Mg, Ca, Mo, and Fe, each in 1.0 μM concentrations, had no detectable stimulatory effect.

Metals removal by conidia before the emergence of germ-tubes appeared to be a rapid surface adsorption rather than an active uptake. When non-germinated conidia (0.5 mg ml^{-1}) were mixed with 0.5 mM Zn, in 0.05 M Tris buffer, pH 7.5, about 80% of the removal occurred in the first minute after mixing; an apparent equilibrium was reached within 10 min (Fig. 2). The extent of metal removal was related to the concentration of conidia. As the number of conidia was increased in the reaction mixture, the amount of free Zn and the amount of Zn bound per conidium decreased (Fig. 3). Binding of the other three metals behaved in a similar fashion (data not shown).

Metal adsorption at biological surfaces is influenced by pH [3,6,21]. Therefore, the effect of pH on metal binding to conidia was studied in the presence of several buffers over a range of pH 3–8. The binding of Fe, Mn, and Zn increased logarith-

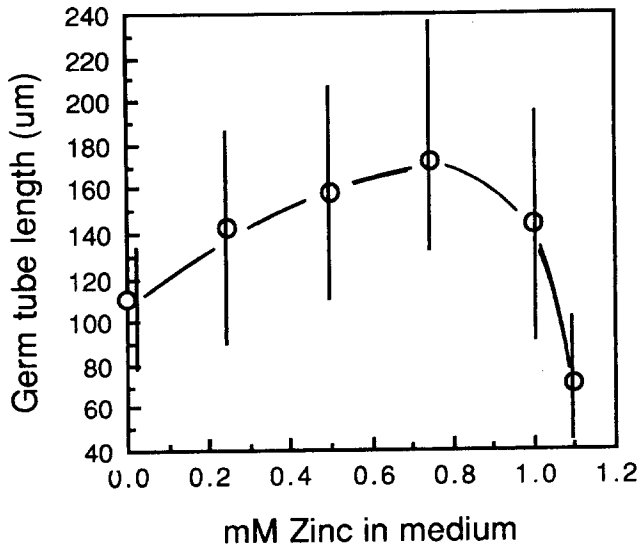

Fig. 1. Efect of Zn concentration on germ tube length measured after 10 h at 26 °C. Each point shows the average and range of measurements on 50 germ tubes.

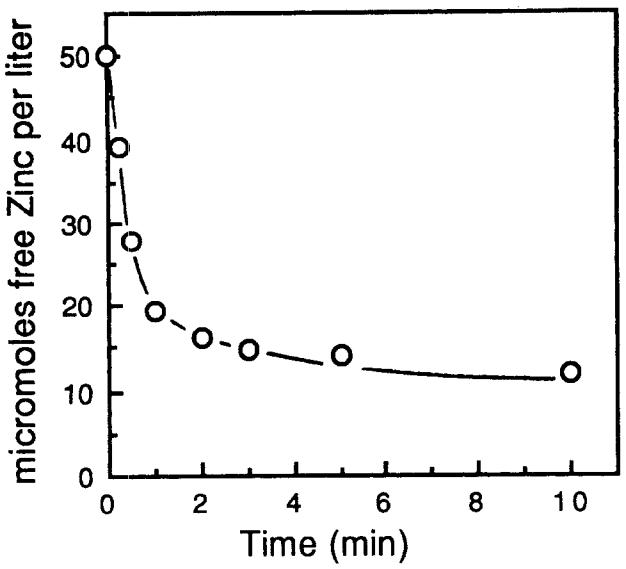

Fig. 2. Time course of free Zn removal by conidia at pH 7.5 in 0.05 M Tris buffer.

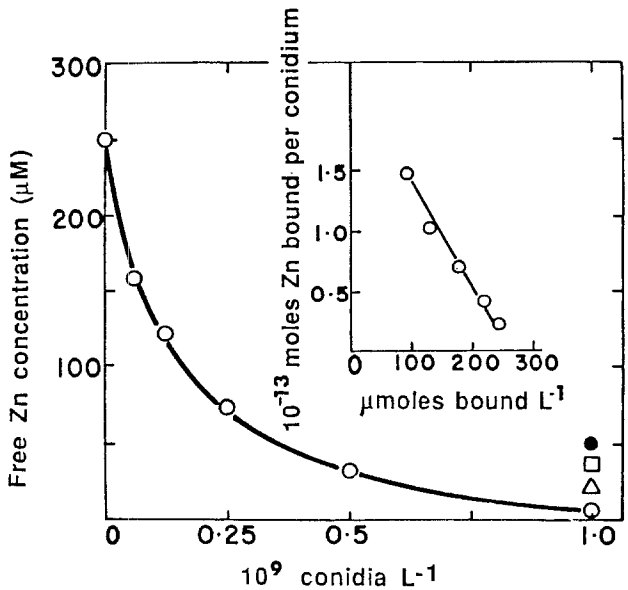

Fig. 3. Effect of conidia concentration on the removal of free Zn from solution. The symbols ●, ❐, Δ, and ○, show comparative removal at pH 4, 5, 6, and 7.5. The inset graph shows the linear decrease in bound Zn per conidium at pH 7.5 as the total amount bound approaches a maximum of 250 μmoles per liter.

mically as the pH was raised (Fig. 4(A)). Anomalous effects were obtained with Fe over pH 5.8 and with Mn and Zn over pH 8, probably due to precipitation of the metals at higher pH values and their removal by filtration. The influence of pH on the binding of copper differed considerably from that of the other three metals, and depended on the buffering system used (Fig. 4(B)). Cu binding increased with pH from 4.5 to 7.3 using cacodylate buffer. When the pH was raised from 4 to 6 with acetate buffer, the adsorption increased in a sigmoidal

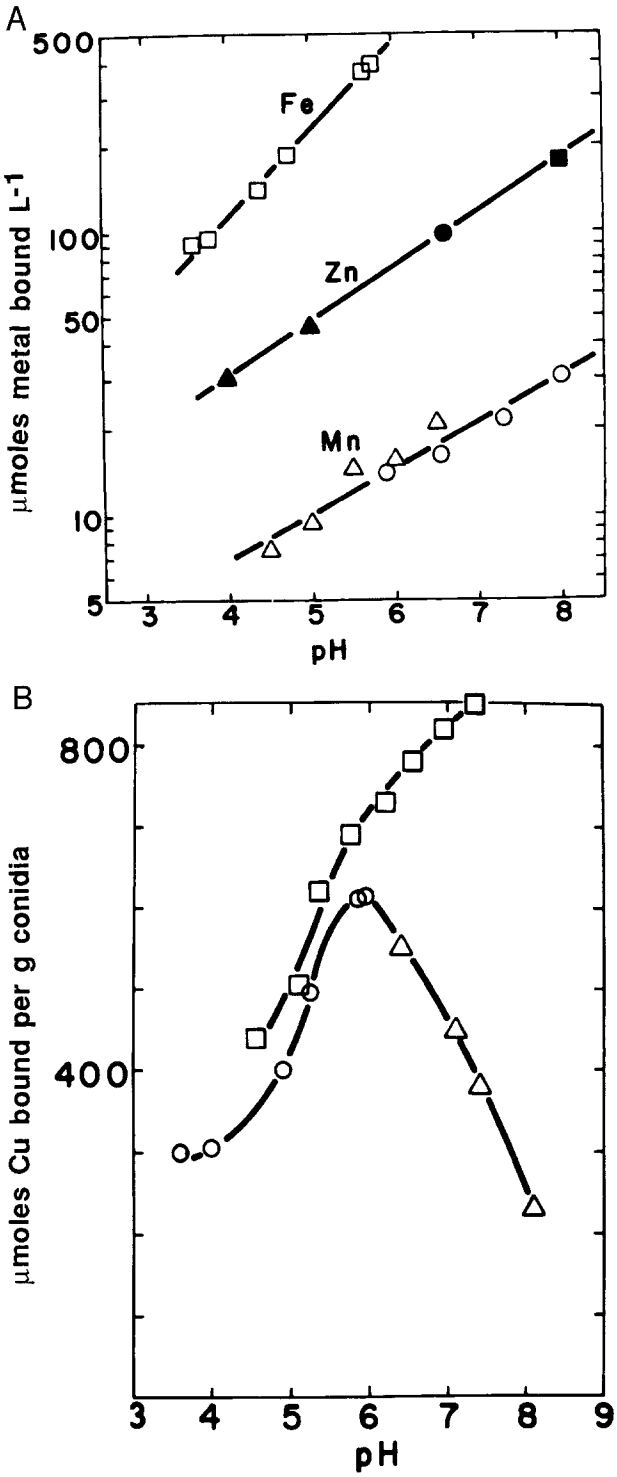

Fig. 4. (A) Binding of Fe, Mn, and Zn at various pH values. Experiments with Fe were performed in mixtures containing 0.1 M acetate buffer, 1 mM Fe, and 0.5 mg conidia ml^{-1}. Experiments with Mn employed mixtures of 0.05 M acetate (Δ) or 0.025 M phosphate (○), 50 μM Mn, and 0.2 mg conidia ml^{-1}. Experiments with Zn employed mixtues of 0.05 M acetate (▲), phosphate (●), or Tris (■) buffers, 0.25 mM Zn, and 0.5 mg conidia ml^{-1}. (B) Effect of pH on copper binding by conidia in 0.1 M acetate buffer (○), 0.1 M Tris (Δ), and in 0.1 M cacodylate buffer (❐). The concentrations of Cu and conidia were 1 mM and 0.5 g L^{-1} respectively.

fashion. Increase in pH from 6.5 to 8.2 caused a progressive decrease in Cu binding to conidial surfaces in Tris buffer.

The effect of ionic strength on metal binding was tested by adding various concentrations of NaCl to binding mixtures. Only Zn was displaced to a detectable extent by NaCl; when the NaCl to $ZnSO_4$ ratio was raised to 20:1, the amount of Zn bound was still 82% of that observed in the absence of added NaCl (Fig. 5).

Studies were also conducted to determine the stability of the metal binding sites on the conidia surfaces when exposed to various enzymes and chemicals. Exposure of stirred conidia for 1 h to chitinase caused small decreases in metal binding capacity (14%, 22%, 9% and 7% for Cu, Fe, Mn and Zn respectively). However, the binding sites were stable to 1 h treatment with protease, beta-glucuronidase, cellulase, and acid phosphatase. The sites were also stable to treatment for 1 h with 0.5 N HCl and 0.5 N NaOH at room temperature. Treatment with lipid solvents (benzene, acetone, and methanol) slightly enhanced (8–12%) the adsorptive capacity of conidia for the metals. The binding sites were stable to boiling for 15 min at pH 7.

The stability of these binding sites on the conidial surfaces prompted experiments to determine if bound metals were released as a result of changes in wall composition during germination. Conidia (1 mg ml^{-1}) were incubated at 26 °C in the aerated germination medium containing 1 mM Zn (chosen as the metal for this experiment because it stimulated germination). The free Zn concentration decreased to 160 μM at 5 min incubation and remained constant for 30 min. At 2 h, a further decrease in free Zn concentration to 21 μM was observed. At that time, the first germ tubes appeared. During the next 18 h, free Zn concentration remained relatively constant (18–29 μM) as the percentage of germination rose to about 80%. Therefore, conidium surface changes concurrent with germination do not cause metal release, nor do the emerging germ tubes contribute to rapid metal binding such as that observed for the conidia. This second conclusion was tested by the addition of 1 mg dry wt of mycelia to a 1 mM Zn solution. The mycelia did not detectably decrease free Zn concentration after 10 min stirring under routine binding conditions. Similarly, soluble concentrations (1 mM) of the other three metals were not detectably lowered by stirring with mycelia.

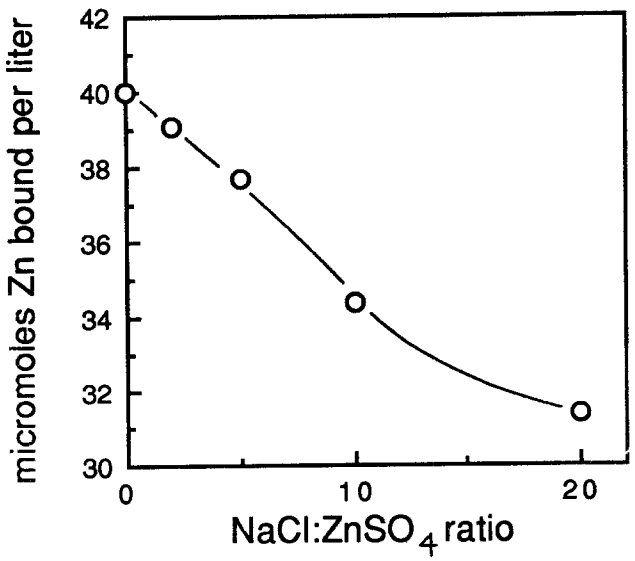

Fig. 5. Effect of ionic strength on the binding of Zn on conidia surfaces. Reaction mixtures contained a total Zn concentration of 500 μM, and 0.5 mg conidia ml^{-1} at pH 7.5 in 0.025 M Tris buffer. Points are averages of data from two experiments.

The binding of the metals by conidia was measured over a 1000-fold range of free metal concentrations to determine conformation to an adsorption isotherm. When the metal binding data were analyzed via Scatchard plots, distinctly inflected curves were obtained such as shown in Fig. 6(A) for Cu binding at pH 5.8. Similar curves were obtained for Fe, Mn, and Zn (data not shown). The binding curves retained their characteristic shapes over the pH range 5–8 as shown in Fig. 6(B) for the binding of Mn at those two pH values. This analysis indicated two classes of binding sites on the conidia surface, one with higher affinity than the other. An equilibrium analysis was performed on binding of the metals at pH 5.8 in 0.1 M acetate buffer, using a 1000-fold range of free metal concentrations. The association constants and respective *n* values for each of the two classes of binding sites are shown in Table 1.

The metals competed with the fungicide, thiabendazole, for sites on the conidial surface. In the absence of added metals, the free concentration of thiabendazole required for half-saturation of the conidial surface at equilibrium was 5.5 μg ml^{-1}. In the presence 1 mM concentrations of Cu, Fe, Mn and Zn, the required thiabendazole concentrations were raised to 21.4, 27.0, 13.9 and 19.2 μg ml^{-1} respectively. No interaction between metals and thiabendazole in the absence of conidia could be detected.

DISCUSSION

The ability of fungi to bind high concentrations of cations has a variety of influences on fungal metabolism and development [reviewed 8,13,18]. The great majority of studies on interactions between metallic cations and fungi have revealed inhibitory effects [reviewed 7], but stimulation of fungal functions by relatively high metal concentrations has rarely been studied aside from the early report by Rothstein [31] that rates of sugar fermentation increased in the presence of high concentrations of metallic cations (Ca^{++}, Mg^{++}, Mn^{++}). These results suggested that titration of fixed anionic groups localized at the cell surface resulted in increased permeability to substrates. Cell wall charge titration might account in part for the stimulation of germ-tube formation and elongation in *P. chartarum* conidia by Zn and Mn at concentrations far above those required as trace elements, but it is puzzling why Fe and Mn would not have a similar effect.

The effects of pH, temperature and chemical treatments on metal binding by *P. chartarum* conidia in our study were quite different from those reported by Galun et al. [16,17] for *Penicillium digitatum* and by Venkateswerlu and Stotzky [37] using cell walls from *Cunninghamella blakesleeana*. Heat and dilute acid/base treatment markedly influenced the metal binding capacities of *P. digitatum* mycelia and *C. blakesleeana*

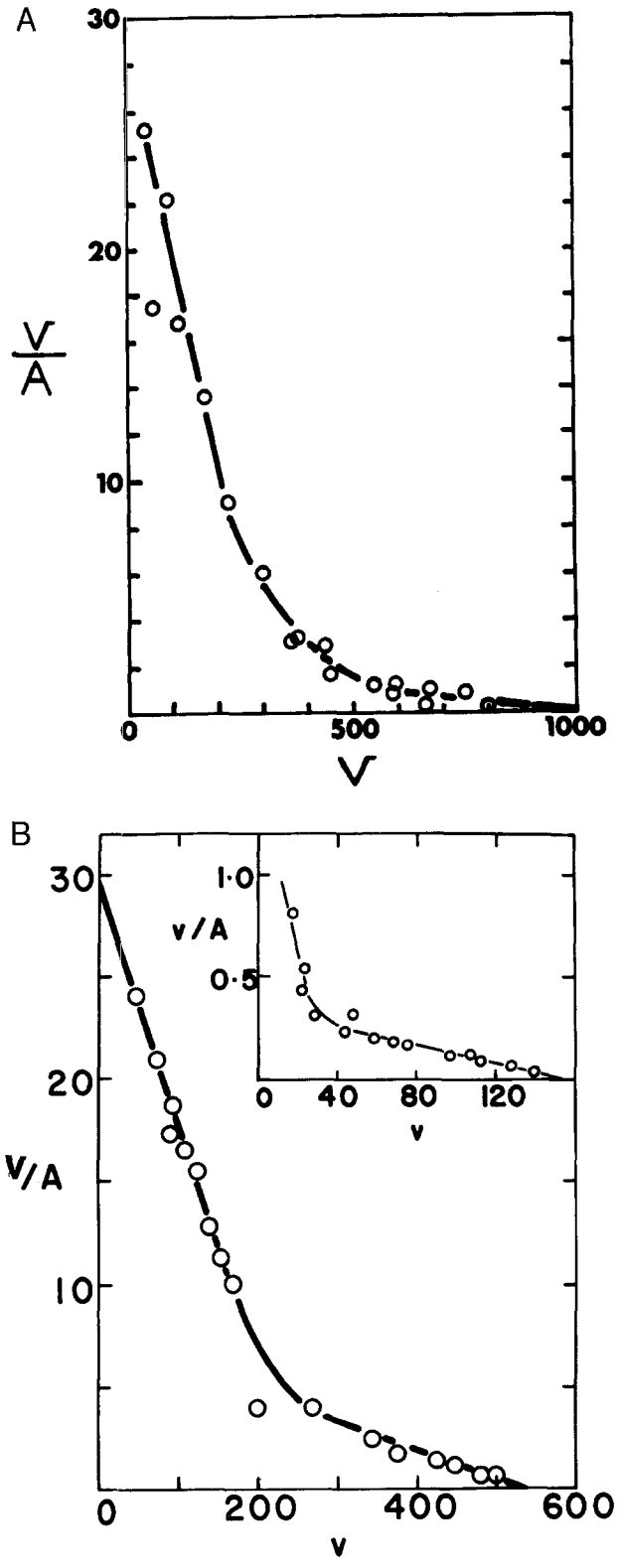

Fig. 6. (A) Scatchard plot of Cu binding to conidia at pH 5.8, in 0.1 M acetate buffer. Values for A are in μmoles per liter; v values are in μmoles bound per g dry wt conidia. The plot is composed of cumulative data from two experiments. (B) Scatchard plot of Mn binding to *Pithomyces* conidia. The main graph illustrates binding at pH 8 in 0.05 M Tris buffer. The inset contains data of binding at pH 5 in 0.1 M acetate buffer. The values for A are in μmoles per liter; v values are in μmoles bound per g dry wt conidia. Each graph is composed of cumulative data from two experiments.

TABLE 1

Association constants and n values for the two classes of metal-binding sites on the surfaces of *P. chartarum* conidia

Metal	K_1	K_2	n_1	n_2
Cu	6.5×10^4	1.9×10^3	339	515
Fe	5.4×10^5	1.4×10^4	188	648
Mn	3.6×10^4	5.0×10^2	26	337
Zn	2.9×10^4	9.3×10^2	145	461

K_1 and K_2 are the association constants of the high affinity and low affinity sites respectively, expressed as liters per mole. The n_1 and n_2 values are the maximal amounts of each metal bound by the high affinity and low affinity sites respectively, expressed as μmoles per g dry wt conidia.

cell walls, but had no effect on *P. chartarum* conidia. Furthermore, unlike *P. digitatum*, the binding of Cu to *P. chartarum* conidia was pH- and buffer-dependent. Decreased binding of Cu to *P. chartarum* conidia in the presence of Tris buffer was probably due to competition between Tris buffer amino groups and Cu-binding sites on the conidia. Cu co-ordinates amines very strongly forming co-ordination spheres consisting of one Cu ion and four amine groups [11,26]. Since Cu co-ordinates with $-NH_2$ but not $-NH_3^+$ groups, it would be expected that as the hydrogen ion concentration decreases, a progressively greater proportion of the Tris molecules would be available for co-ordination.

The magnitude of metal ion binding to the *P. chartarum* conidial surface can be estimated as follows. The approximate dimensions of a conidium are 10×15 μm [24]. If, for the sake of simplicity in calculation, its shape were assumed to be a smooth, symmetrical ellipsoid, the surface area could be estimated to be about 450 μm^2 by the formula: $4/3\ \pi (a^2 + b^2 + c^2)$, where a, b and c are the radii of the three axes of the ellipsoid. Using an average ionic raidus of 0.8 Å for the four metal ions [25] and a value of 3.3×10^9 conidia per gram having a total surface area of 1.5×10^{20} Å^2, at saturation levels, Cu would be deposited on the spore surface in a continuous layer about 5 ions thick. For Fe, Mn and Zn, the multiplicity of layering would be (to the nearest whole layer) 5, 2 and 4 ions thick respectively. This calculation does not, of course, take into account the porosity of the wall matrix and the irregularities of the conidial surface. Although the *Pithomyces* conidia surface area is further extended by fine, rod-like spicules (800 nm by 40 nm) attached to the surface at one end and standing out at right angles [4], they apparently do not contribute to metal binding, since benzene treatment of conidia (which removes the spicules) slightly increased metal adsorptive capacity of the conidial surface.

Although mycelial surfaces have far less metal binding capacity than those of spores from the same species [15,23], it was unexpected that *Pithomyces* mycelia would have no detectable ability to bind the four metals used in the present study. The gross composition of the conidium wall in *Pithomyces* is similar to that of the mycelial wall except for its lower carbohydrate and higher protein content; furthermore,

the two glycoprotein fractions isolated from conidial wall material had the same sugar and amino acid composition as the corresponding glycoproteins similarly extracted from mycelia [33,34]. Mycelia of other fungi have metal binding capacities of 40–1900 μmol g^{-1} dry wt [30,37], a range which brackets those observed here for *P. chartarum* conidia.

In metal binding studies with most fungi [16,21,28,30], the binding of metal ions to cell surfaces appeared to obey a simple adsorption isotherm. However, our binding data, obtained over a 1000-fold range of free metal concentrations, indicated at least two classes of sites. It is interesting in that regard that the widely-used substituted benzimidazole fungicides (such as benomyl and thiabendazole) also saturate conidial surfaces by binding at two sites with different affinities [35]. These benzimidazoles avidly adsorb to the surfaces of dormant conidia, and are released during germination to provide a high concentration of free fungicide in the region of the emerging germtube [36]. Metal ions compete with fungicide for available surface sites; their presence in high concentrations (which occur in the warm, moist environment of decomposing organic matter favoring the rapid growth of this saprophytic fungus [1,10,20]) reduces the ability of conidia to accumulate fungicide and hence would compromise its effectiveness in the field.

ACKNOWLEDGEMENTS

The author thanks Owen Clinton for metal analysis and Lyndon Larcom for informative discussion.

REFERENCES

1 Alexander, M. 1977. Introduction to Soil Microbiology (2nd edn), pp. 368–401, John Wiley and Sons, New York.
2 Aronson, J.M. 1981. Cell wall chemistry, ultrastructure and metabolism. In: Biology of Conidial Fungi (Cole, G.T. and B. Kendrick, eds), pp. 459–505, Academic Press, New York.
3 Bauda, P. and J.C. Block. 1990. Role of envelopes of Gram-negative bacteria in cadmium binding and toxicity. Toxic. Assess. 5: 47–60.
4 Bertaud, W.S., I.M. Morice, D.W. Russell and A. Taylor. 1963. The spore surface in *Pithomyces chartarum*. J. Gen. Microbiol. 32: 285–395.
5 Brierley, C.L., J.A. Brierley and M.S. Davidson. 1989. Applied microbial processes for metals recovery and removal from wastewater. In: Metal Ions and Bacteria (Beveridge, T.J. and R.J. Doyle, eds), pp. 359–382, John Wiley and Sons, New York.
6 Campbell, P.G.C. and P.M. Stokes. 1985. Acidification and toxicity of metals to aquatic biota. Can. J. Fish. Aquat. Sci. 42: 2034–2049.
7 Collins, Y.E. and G. Stotzky. 1989. Factors affecting the toxicity of heavy metals to microbes. In: Metal Ions and Bacteria (Beveridge, T.J. and R.J. Doyle, eds), pp. 31–90, John Wiley and Sons, New York.
8 Cotter, D.A. 1980. Spore activation. In: The Fungal Spore: Morphogenetic Controls (Turian, G. and H.R. Hohl, eds), pp. 385–411, Academic Press, London.
9 deRome, L. and G.M. Gadd. 1991. Use of pelleted and immobilized yeast and fungal biomass for heavy metal and radionuclide recovery. J. Ind. Microbiol. 7: 97–104.
10 diMenna, M.E. and J.R. Bailey. 1973. *Pithomyces chartarum* spore counts in pasture. N.Z. J. Agric. Res. 16: 343–351.
11 Edsall, J.T. and J. Wyman. 1958. Biophysical Chemistry, vol. 1. Academic Press, New York.
12 Feldman, H.A. 1972. Mathematical theory of complex ligand-binding systems at equilibrium: some methods for parameter fitting. Anal. Biochem. 48: 317–338.
13 Gadd, G.M. 1992. Molecular biology and biotechnology of microbial interactions with organic and inorganic heavy metal compounds. In: Molecular Biology and Biotechnology of Extremophiles (Herbert, R.A. and R.J. Sharp, eds), pp. 225–257, Blackie & Sons, Glasgow.
14 Gadd, G.M. 1993. Interactions of fungi with toxic metals. New Phytol. 124: 25–60.
15 Gadd, G.M. and J.L. Mowll. 1985. Copper uptake by yeast-like cells, hyphae and chlamydospores of *Aureobasidium pullulans*. Exp. Mycol. 9: 230–240.
16 Galun, M., E. Galun, B.Z. Siegel, P. Keller, H. Lehr and S.M. Siegel. 1987. Removal of metal ions from aqueous solutions by *Pencillium* biomass: kinetic and uptake parameters. Water, Air and Soil Pollution 33: 359–371.
17 Galun, M., P. Keller, D. Malki, H. Feldstein, H. Galun, S.M. Siegel and B.Z. Siegel. 1983. Removal of uranium (VI) from solution by fungal biomass and fungal wall-related biopolymers. Science 219: 285–286.
18 Hassall, K.A. 1990. The Biochemistry and Use of Pesticides. 2nd edn, pp. 266–325, VCH Publishers, New York.
19 Hunt, S. 1986. Diversity of biopolymer structure and its potential for ion-binding applications. In: Immobilization of Ions by Biosorption (Eccles, H. and S. Hunt, eds), pp. 16–46, Ellis Harwood, Chichester, UK.
20 Jackson, M.L. 1964. Chemical composition of soils. In: Chemistry of the Soil (Bear, F.E., ed.), pp. 71–141, Reinhold Co., New York.
21 Kuyucak, N. and B. Volesky. 1988. A method of metal removal. Wat. Poll. Res. J. Can. 23: 424–433.
22 Moore-Landecker, E. 1990. Fundamentals of the Fungi. pp. 6–7, Prentice-Hall, Englewood Cliffs, NJ.
23 Mowll, J.L. and G.M. Gadd. 1984. Cadmium uptake by *Aureobasidium pullulans*. J. Gen. Microbiol. 130: 279–284.
24 Muller, E. and W. Loeffler. 1976. Mycology. pp. 275–276, Georg. Thieme Publishers, Stuttgart.
25 Nebergall, W.H., F.C. Schmidt and H.F. Holtzclaw. 1972. General Chemistry (4th edn), pp. 816–911, D.C. Heath and Co., Lexington, MA.
26 Orgel, L.E. 1958. Enzyme-metal-substrate complexes as coordination compounds. In: Metals and Enzyme Activity (Crook, E.M., ed.), pp. 10–14, Cambridge Univ. Press, London.
27 Parle, J.E. and M.E. diMenna. 1972. Fungicides and the control of *Pithomyces chartarum*. N.Z. J. Agric. Res. 15: 54–63.
28 Paton, W.H.N. and K. Budd. 1972. Zinc uptake in *Neocosmospora vasinfecta*. J. Gen. Microbiol. 72: 173–184.
29 Pumpel, T. and F. Schinner. 1991. Native fungal pellets as a biosorbent for heavy metals. In: European Federation of Biotechnology IX International Symposium, Biohydrometallurgy '91 (Duarte, J.C. and R.W. Lawrence, eds), pp. 49–51, FORBITEC Editions, Troia, Portugal.
30 Ross, I.S. and C.C. Townsley. 1986. The uptake of heavy metals by filamentous fungi. In: Immobilization of Ions by Bio-sorption (Eccles, H. and S. Hunt, eds), pp. 49–58, Ellis Harwood, Chichester, UK.
31 Rothstein, A. 1955. Relationship of the cell surface to electrolyte metabolism in yeast. In: Electrolytes in Biological Systems (Shanes, A.M., ed.), p. 88, American Physiological Society, Washington, DC.

32 Scatchard, G. 1949. The attraction of proteins for small molecules and ions. Ann. New York Acad. Sci. 51: 660–672.
33 Sturgeon, R.J. 1964. Components of the cell wall of *Pithomyces chartarum*. Proc. Biochem. Soc. 4: 60–61.
34 Sturgeon, R.J. 1966. Components of the spore wall of *Pithomyces chartarum*. Nature 206: 204.
35 Stutzenberger, F.J. and J.N. Parle. 1972. Binding of benzimidazole compounds to conidia of *Pithomyces chartarum*. J. Gen. Microbiol. 73: 85–94.
36 Stutzenberger, F.J. and J.N. Parle. 1973. Binding of 2-(2-oxazolyl) benzimidazole by *Pithomyces chartarum* conidia and release during germination. J. Gen. Microbiol. 78: 199–201.
37 Venkateswerlu, G. and G. Stotzky. 1989. Binding of metals by cell walls of *Cunninghamella blakesleeana* grown in the presence of copper or cobalt. Appl. Microbiol. Biotechnol. 31: 619–625.