Copper Uptake by *Penicillium ochro-chloron*: Influence of pH on Toxicity and Demonstration of Energy-dependent Copper Influx Using Protoplasts

By G. M. GADD* and C. WHITE

Department of Biological Sciences, University of Dundee, Dundee DD1 4HN, UK

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The existence of energy-dependent copper influx was demonstrated in protoplasts of *Penicillium ochro-chloron*. Protoplasts and mycelium were tolerant of copper over the pH range 3.0 to 5.5 but sensitive at pH 6.0. Uptake of copper was approximately ten times greater at pH 6.0 than in the lower range. At pH 3.0, influx showed saturation kinetics with a half-maximal influx at an external Cu\(^{2+}\) concentration of 390 μM and a maximum influx rate of 22 nmol h\(^{-1}\) (10\(^7\) cells\(^{-1}\)). Saturation kinetics were not observed at pH 6.0.

**INTRODUCTION**

*Penicillium ochro-chloron* is a fungus possessing a high tolerance to copper and has been isolated from copper-rich soils and copper-plating solutions (Okamoto & Fuwa, 1974; Stokes & Lindsay, 1979). In growth media, a constant copper uptake occurs above external concentrations of approximately 16 mM (Okamoto et al., 1977) and previous work has shown that intracellular concentrations of low molecular weight organic solutes, particularly glycerol, may have a significant role in protection from copper and salt stress (Gadd et al., 1984a). However, although *P. ochro-chloron* is reported to have a high growth requirement for copper (Basu et al., 1955), there has been no demonstration of energy-dependent uptake. Energy-dependent influx of metal ions, like Ni\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\) and Cu\(^{2+}\), has been shown in some filamentous fungi but, apart from *Neurospora crassa* (Mohan et al., 1984), there is scant information relating to other species. More attention has been paid to yeasts and yeast-like fungi which possess energy-dependent influx systems for various metal ions (Fuhrmann & Rothstein, 1968; Norris & Kelly, 1977; Borst-Pauwels, 1981; Mowll & Gadd, 1983, 1984). The paucity of information relating to filamentous fungi partly arises from problems associated with the mycelial growth habit. Preparation of a homogeneous suspension is difficult and there may be difficulties in removing free ions trapped in interhyphal spaces. In addition, high wall binding may mask low rates of intracellular influx. High wall binding of metal ions also occurs with yeast but amounts of metal subsequently taken up by energy-requiring processes are much higher and can easily be distinguished from wall adsorption (Norris & Kelly, 1977; Mowll & Gadd, 1983). In fungi other than yeasts, influx systems seem to be of low affinity and in *Aureobasidium pullulans*, sensitive radioisotopic methods proved best for analysis (Mowll & Gadd, 1984).

Protoplasts may provide a more homogeneous experimental system for the study of metal uptake by filamentous fungi and, in the absence of the cell wall, influx will not be obscured by wall binding. This paper demonstrates the potential of protoplasts for such investigations and, in addition, attention is paid to the relationship of copper influx to toxicity and the influence of pH.

**METHODS**

*Organism and culture conditions. Penicillium ochro-chloron* (IMI 39806) was maintained on malt extract agar (Oxoid) and in liquid medium comprising (g l\(^{-1}\)): glucose, 20.0; (NH\(_4\))\(_2\)SO\(_4\), 5.0; KH\(_2\)PO\(_4\), 0.5; MgSO\(_4\) \(_7\)H\(_2\)O, 0.2; CaCl\(_2\), 2H\(_2\)O, 0.5; NaCl, 0.1; FeCl\(_3\), 0.0025; ZnSO\(_4\) \(_7\)H\(_2\)O, 0.004; MnSO\(_4\) \(_4\)H\(_2\)O, 0.004; CuSO\(_4\) \(_5\)H\(_2\)O,

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0.0004. For experimental cultures, 100 ml medium was inoculated with 1.0 ml from a 96 h starter culture and grown for 40 h at 25°C on an orbital shaker (100 cycles min⁻¹).

Mycelium was harvested by filtration with 63 μm aperture nylon mesh (Staniar, Sherborne St, Manchester M3 1FD, UK), washed twice with distilled water and, after removal of excess water by blotting with absorbent paper, was weighed.

Protoplast isolation. Harvested mycelium was washed in buffer comprising 100 mM-MES with 0.8 M-KCl, pH 5-8, resuspended in 100 ml of the same buffer containing 10 mg Novozym 234 ml⁻¹ (Novo Enzyme Products, 2B Thames Ave, Windsor SL4 1QP, UK) to a density of 0.05 g wet wt ml⁻¹ and then incubated for 35 min at 25°C with stirring. The crude protoplast preparation was separated by centrifugation (1200 g for 3 min) using a swing-out head, repeating if necessary, and resuspended in 20 ml of a buffer comprising 20 mM-MES with 0.8 M-KCl, pH 6-0. Mycelial remnants were removed by filtration through a 35 μm aperture nylon mesh, washing through with the above buffer as necessary (200-300 ml) and then by passing the resulting filtrate through a 20 μm mesh. Protoplasts were removed from this buffer by centrifugation (1200 g for 5 min), washed in 1-2 M-sorbitol (about 20 ml) and resuspended in 1-2 M-sorbitol.

Respirometry. Mycelium (approximately 10 mg wet wt ml⁻¹) or protoplasts (approximately 1.5 × 10⁷ ml⁻¹) were incubated at 25°C on an orbital shaker (100 cycles min⁻¹) in a medium comprising 1.2 M-sorbitol, glucose and 50 mM-potassium hydrogen phthalate with or without 50 mM-CuSO₄. The pH was adjusted using tetramethylammonium hydroxide. Respiration rates of 2 ml aliquots were determined after 80 min for protoplasts and 330 min for whole mycelium (times needed to give about 50% inhibition of respiration in 10 mM-copper at pH 6) using a Clark-type oxygen electrode (Rank Bros, Bottisham, Cambridge CB5 9DA, UK).

Copper uptake by protoplasts. Protoplasts were suspended to a density of approximately 5 × 10⁷ ml⁻¹ in a buffer comprising 50 mM-potassium hydrogen phthalate with 1.2 M-sorbitol, adjusted to the required pH using tetramethylammonium hydroxide and incubated for 1 h at 25°C with stirring. Glucose was added (final concentration 50 mM) and the mixture incubated for a further 30 min. KCN (final concentration 0.2 mM) where used, was added after 25 min incubation with glucose.

Irradiated ⁶⁴Cu (half-life 12.9 h) as CuSO₄ was obtained from the Scottish Universities Research and Reactor Centre, East Kilbride. This was added as a concentrated stock solution in 1.2 M-sorbitol to the cell suspension to initiate uptake. Activities were in the range 0.2 to 22.8 kBq ml⁻¹. The maximum copper concentration used in uptake experiments, at pH 3 or pH 6, was 1.0 mM as higher concentrations would require adjustment of the pH following copper addition, which was undesirable for safety reasons. Samples were removed at intervals and separated from the uptake medium by microcentrifugation through a cushion (0.5 ml) of the incubation buffer containing 3 M (w/v) Nycodenz, a density gradient material (Nyegaard (UK), Birmingham B26 3DU, UK) (Gadd et al., 1984b). After removal of the supernatant, protoplast pellets were resuspended in 100 μl 40% (v/v) perchloric acid and counted for radioactivity as previously described (Mowll & Gadd, 1984).

Other methods. Protoplast numbers were assessed using a modified Fuchs–Rosenthal haemocytometer. Dry weights of mycelium were determined using tared aluminium foil cups dried to constant weight at 105°C.

Chemicals. Except where otherwise stated, chemicals were of analytical or similar grade.

RESULTS

The protoplast preparations obtained were substantially free from walled fragments and debris as determined by phase-contrast microscopy and the yield was approximately 3.5 × 10⁵ protoplasts (mg wet wt of mycelium)⁻¹. P. ochro-chloron protoplasts accumulated copper from solution in a manner consistent with metabolic dependence, uptake being stimulated by glucose and inhibited by KCN and incubation at 4°C (Fig. 1). Copper uptake was concentration-dependent at both pH 3.0 and 6.0 but overall values were greater at pH 6.0 (Fig. 2). Furthermore, at pH 3.0 influx approximated to saturation kinetics, with a concentration for half-maximal influx of 390 μM and a maximal influx of 22 nmol h⁻¹ (10⁵ cells)⁻¹. At pH 6.0 there was distinct deviation from saturation kinetics and at external copper concentrations above 200 μM, uptake was approximately linear with concentration. Below this concentration, results did not permit analysis of kinetics.

Uptake was unaffected by pH at values between 3.0 and 5.5 (Table 1) and copper had no inhibitory effect on the respiration of either protoplasts or whole mycelium at the concentrations and conditions used. However, where influx was greatly increased at pH 6.0, marked toxicity, as indicated by inhibition of respiration, occurred (Table 1). The onset of toxicity, however, was delayed in whole mycelium compared with protoplasts; 50% inhibition occurred after 338 min rather than 78 min.
Copper uptake by Penicillium ochro-chloron

Fig. 1. Copper uptake by *P. ochro-chloron* protoplasts at pH 6.0 from an external copper concentration of 10 μM. ○, 50 mM-glucose; ●, no glucose; □, 50 mM-glucose + 0.2 mM-KCN; ■, 50 mM-glucose at 4 °C (all others at 25 °C). Bars denote SE.

Fig. 2. Effect of external copper concentration on copper uptake by *P. ochro-chloron* protoplasts. The glucose concentration was 50 mM. Uptake is total uptake over 1 h. ○, pH 3.0; ●, pH 6.0. Bars denote SE.

Table 1. Copper uptake by protoplasts and the effect of copper on respiration rates of protoplasts and whole mycelium of *P. ochro-chloron* at various pH values

<table>
<thead>
<tr>
<th>pH</th>
<th>Copper uptake* (nmol h⁻¹ (10⁷ cells)⁻¹)</th>
<th>Respiration rate (percentage of control rate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Protoplasts</td>
</tr>
<tr>
<td>3.0</td>
<td>5.1 ± 0.48</td>
<td>100.0 ± 13.1</td>
</tr>
<tr>
<td>3.5</td>
<td>2.9 ± 0.91</td>
<td>100.0 ± 9.3</td>
</tr>
<tr>
<td>4.0</td>
<td>3.8 ± 0.59</td>
<td>103.9 ± 4.5</td>
</tr>
<tr>
<td>4.5</td>
<td>6.7 ± 0.90</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>5.0</td>
<td>3.0 ± 0.50</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>5.5</td>
<td>3.7 ± 0.09</td>
<td>100.0 ± 13.2</td>
</tr>
<tr>
<td>6.0</td>
<td>38.6 ± 6.0</td>
<td>45.5 ± 9.6</td>
</tr>
</tbody>
</table>

* Total uptake after 1 h.

**DISCUSSION**

The use of protoplasts has permitted the demonstration of the existence of energy-dependent copper influx in *P. ochro-chloron*. Energy-dependent influx of heavy metal ions has been demonstrated in *Neurospora crassa* (Venkateswerlu & Sastry, 1970; Mohan *et al.*, 1984) and *Neocosmospora vasiforme* (Paton & Budd, 1972) but there is little work on other filamentous fungi. By applying accumulation data to adsorption isotherms it was concluded that lead, cadmium and zinc uptake by *Pythium* sp., *Scytalidium lignicola* and *Dictyuchus sterile* was largely an adsorptive process (Duddridge & Wainwright, 1980). However, metal ion concentrations
were low (<0.15 mM) and complete removal of ions from solution or masking of influx by wall-binding may have occurred. It should be stressed that filamentous fungi must have influx mechanisms for cellular entry of essential metal ions such as copper and zinc. The use of protoplasts eliminates interference caused by wall-binding and allows low rates of influx to be detected even at low external ion concentrations. Adequate protoplast yields can be obtained from fungi with Novozym 234 or other commercially available enzyme preparations (Hamlyn et al., 1981; White & Gadd, 1984), which, as this paper demonstrates, are amenable to sensitive radioisotopic and microcentrifugation methods (Mowll & Gadd, 1983, 1984). We have previously demonstrated the use of protoplasts for assessing the relative contributions made by the cell-wall and membrane transport to total copper uptake in Saccharomyces cerevisiae (Gadd et al., 1984b).

Copper uptake and toxicity were essentially constant over the pH range 3.0 to 5.5, which is in agreement with Stokes & Lindsay (1979), but at pH 6.0 increased uptake and toxicity were evident. The connection between reduced uptake at low pH and low toxicity is clear for Scytalidium where the pH range 4.2 to 5.0 was critical (Starkey, 1973) while for a strain of Aureobasidium pullulans, copper uptake and toxicity were markedly reduced at low pH. In growth experiments, decreased copper uptake and alleviation of toxicity coincided with the fall in pH of the medium (Gadd & Griffiths, 1980). Toxicity reduction with increased acidity has not been observed in all fungi (Starkey, 1973; Babich & Stotzky, 1981) although some pH effects can be accounted for by the chemical behaviour of the ionic species involved, for example the formation of insoluble hydroxides at pH values around neutrality.

At pH 6.0 and at copper concentrations greater than 200 μM, copper influx deviated from the saturation kinetics generally found for energy-dependent influx processes (Fuhrmann & Rothstein, 1968; Paton & Budd, 1972; Norris & Kelly, 1977; Mowll & Gadd, 1983, 1984), which suggested the existence of an additional uptake component, possibly as a consequence of damage to the membrane by copper at this pH. At lower copper concentrations this deviation was not apparent although the small number of datum points did not permit adequate analysis.

At pH 3.0, the relationship of copper uptake to the external copper concentration closely paralleled that found in growing cultures (Okamoto & Fuwa, 1974; Gadd et al., 1984a), which supports the view that the low copper uptake seen in these studies depends on low transport rates across the membrane. Since this property has been demonstrated in protoplasts it cannot be due primarily to the cell wall acting as a permeability barrier but must result mainly from properties of the uptake system. However, the cell wall may also play some role in tolerance as the respiration of protoplasts was more rapidly affected by copper than that of whole mycelium.

This study has demonstrated that isolated protoplasts provide a useful tool for the elucidation of uptake and the responses of filamentous fungi to heavy metal ions, both technically in providing homogeneous and easily manipulated preparations and in allowing evaluation of the contributions of the wall and the membrane transport systems to total uptake.

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