The Osmotic Responses of *Penicillium ochro-chloron*: Changes in Internal Solute Levels in Response to Copper and Salt Stress

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The marked copper tolerance of *Penicillium ochro-chloron* has been confirmed as has its ability to grow in solutions of high salinity. The major low molecular weight organic solutes present in *P. ochro-chloron* were glycerol, erythritol, arabitol, mannitol, sorbitol and trehalose. Of these, glycerol increased significantly during growth in high concentrations of Na⁺ or Cu²⁺, a 15-fold increase, relative to the control, occurring in concentrations of 0.5 M. Cell K⁺ increased with elevated external Na⁺ but decreased slightly with elevated external Cu²⁺. With high external Cu²⁺ mycelium maintained a constant level of Cu²⁺, with low levels of Na⁺ in all treatments. It is concluded that the high concentrations of glycerol induced by high external Na⁺ or Cu²⁺ had a significant osmotic effect, allowing growth in media of high osmolality. The exclusion of ions may have a gratuitous role in the copper tolerance of *P. ochro-chloron*.

INTRODUCTION

Above micronutrient levels, the fungicidal nature of copper compounds is commonly assumed, but certain fungi have the ability to withstand and grow in concentrations that are toxic to most other organisms (see Ross, 1975; Gadd & Griffiths, 1978). *Penicillium ochro-chloron* has been the subject of a number of studies because of its extreme tolerance to copper and certain other metals. It has a high copper requirement for optimal growth and sporulation (Basu *et al.*, 1955) and can grow in copper sulphate concentrations up to saturation (Okamoto & Fuwa, 1974; Okamoto *et al.*, 1977). There are widely differing values reported for copper accumulation by this organism (Basu *et al.*, 1955; Okamoto & Fuwa, 1974; Stokes & Lindsay, 1979; Baldry & Dean, 1980), but, as yet, there is no detailed information on the physiology of heavy metal tolerance. Resistance mechanisms found in some other fungi, such as sulphide or oxalate production or intracellular localization (see Ross, 1975; Gadd & Griffiths, 1978; Murphy & Levy, 1983), have not been demonstrated in *P. ochro-chloron*.

Copper sulphate solutions are acidic and while low pH values are effective in moderating copper toxicity to *Scytalidium* sp. (Starkey, 1973) and *Aureobasidium pullulans* (Gadd & Griffiths, 1980), for *P. ochro-chloron* there is little difference in the effects of copper on growth from pH 2.0 to pH 6.0 (Stokes & Lindsay, 1979).

Fungi growing in solutions of high osmolality often accumulate one or more intracellular solutes that have an 'osmoregulatory' function (see Jennings, 1983). Since a considerable osmotic potential is generated by high copper sulphate concentrations (1.129 M-CuSO₄ ≡ 0.523 M-NaCl ≡ sea water ≡ 1.05 osmol kg⁻¹), the intracellular solute content of *P. ochro-chloron* was investigated over a range of external copper concentrations to establish the role, if any, of such solutes in heavy metal tolerance. Growth and intracellular solute concentrations have also been studied in response to NaCl stress to determine whether similar osmotic responses are initiated by NaCl and CuSO₄.

Natural abundance ¹³C nuclear magnetic resonance spectrometry (NMR) was used to identify the major low molecular weight organic solutes in *P. ochro-chloron*. This technique

allows unequivocal identification and, where these organic solutes are present in osmotically significant amounts, the ^{13}C resonances from such compounds can be identified in a relatively short time (Borowitzka *et al.*, 1980; Reed *et al.*, 1984). Intracellular accumulation of low molecular weight organic solutes (quantified by gas-liquid chromatography) was coupled to measurements of the principal inorganic ions to assess the osmotic significance of inorganic and organic solutes in *P. ochro-chloron*.

METHODS

Organism, media and growth conditions. *Penicillium ochro-chloron* (IMI 39806) was routinely maintained on 4.7% (w/v) malt agar (Lab M. Ford Lane, Salford, UK). The liquid medium used was of composition (g l^{-1}): D-glucose, 20.0; $(\text{NH}_4)_2\text{SO}_4$, 5.0; KH_2PO_4 , 0.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05; NaCl, 0.1; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.0025; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.004; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0004. Starter cultures were prepared by inoculating 50 ml culture medium with several 6 mm diameter discs excised from actively growing colonies on malt agar and incubating for 24–28 h on an orbital shaker (100 cycles min^{-1}). These were used, after one further subculture, to inoculate aspirators containing 1 litre medium to an initial density of approximately 0.04 mg dry wt ml^{-1} . Required amounts of crystals of CuSO_4 or NaCl (sterilized at 160 °C for at least 3 h) were added to the aspirators after sterilization. All cultures were incubated at 23 °C with stirring and forced aeration.

^{13}C NMR. Natural abundance $^{13}\text{C}\{^1\text{H}\}$ NMR spectra were measured using a Bruker WP60 FT spectrometer operating at 15.08 MHz. D_2O (10%) was added to each sample to provide a lock signal and methanol [49.0 p.p.m. downfield from 3-(trimethylsilyl)-1-propanesulphonic acid] was used as an internal standard. Spectra were obtained using a 7 μs pulse (65°) and a recycle time of 1.1 s. A total of 1500 scans was accumulated for each sample; these were transformed after exponential broadening of 1.5 Hz. Concentrated extracts of 2-week-old mycelium were obtained by extraction in 80% (v/v) ethanol overnight, and were assayed at 30 °C in 10 mm o.d. tubes.

Gas-liquid chromatography. GLC separation of the major low molecular weight, alcohol-soluble organic solutes was carried out on trimethylsilyl-derivatized samples, following extraction in 80% (v/v) ethanol, rotary evaporation and storage in a vacuum desiccator for 48 h (Holligan & Drew, 1971). Samples were assayed in pyridine using a Varian 3700 chromatograph and Hewlett Packard 3390A integrator, with the temperature programmed from 90 °C to 280 °C at 20 °C min^{-1} , holding the initial and final temperatures for 1 min. All other conditions were as previously described (Reed *et al.*, 1980; Reed & Stewart, 1983).

Metal analysis. Samples (10 ml) of mycelial suspensions were harvested in the stationary phase after 12–14 d growth by vacuum filtration onto Whatman GF/C glass microfibre papers (5.5 cm diameter), rinsed with distilled water and then washed twice with 0.5 M- CaCl_2 , to remove loosely associated extracellular ions, at 4 °C. Mycelial mats were easily removed from the filter papers by peeling and placed in clean polypropylene tubes. For ion extraction, 2.0 ml 6 M- HNO_3 was added to the pellets, which were then kept at 90 °C for 2 h. Distilled water (8.0 ml) was then added and the extracts were left to settle overnight. Ion concentrations in these supernatants and reagent controls were determined using a Pye Unicam SP9 atomic absorption spectrophotometer after appropriate dilution.

Other methods. Dry weights were determined using tared aluminium foil cups dried to constant weight at 105 °C. Intracellular water content was measured as: total fresh wt – dry wt – extracellular water content. Here, dry weights were measured after drying to constant weight at 65 °C for 7 d. Extracellular water contents were assayed by short-term incubation (120 s) in [^{14}C]sorbitol (10 mM; specific activity 2 GBq mol^{-1}), as described by Reed (1980).

RESULTS

Natural abundance ^{13}C NMR spectroscopy revealed the major low molecular weight organic solutes present in *P. ochro-chloron* and representative traces are shown in Fig. 1. The traces, as shown, give no quantitative data, although the relative importance of the principal organic solutes can be seen by studying the lines corresponding to each compound, in relation to each other. The spectra are more complex than previously published spectra for cyanobacteria (Borowitzka *et al.*, 1980; Mackay *et al.*, 1983; Reed *et al.*, 1984), due to the presence of several low molecular weight organic solutes (see Hocking & Norton, 1983), namely glycerol (δ 63.8 [C-1/C-3] and 73.2 [C-2]), erythritol (δ 63.8 [C-1/C-4] and 73.2 [C-2/C-3]), arabitol (δ 64.2 [C-1/C-5], 71.4 [C-2/C-3] and 72.2 [C-4]), mannitol (δ 64.6 [C-1/C-6], 72.2 [C-2/C-5] and 70.7 [C-3/C-4]), sorbitol (δ 63.7 [C-1], 72.3 [C-2/C-4], 71.0 [C-3], 74.2 [C-5] and 64.1 [C-6]) and trehalose (δ 94.5 [C-1], 73.6 [C-2/C-3], 70.8 [C-4], 72.3 [C-5] and 61.9 [C-6]). Since the resonances are often

Fig. 1. ^{13}C NMR spectra of mycelial extracts of *P. ochro-chloron*. (a) Control; (b) 0.5 M-NaCl; (c) 0.5 M- CuSO_4 . The peaks are labelled as follows: a, arabitol; e, erythritol; g, glycerol; m, mannitol; s, sorbitol; t, trehalose.

superimposed, interpretation of the traces is complex, although most compounds have at least one unique resonance by which they can be identified (Fig. 1). Unidentified, smaller resonances were also observed at δ 76.4, 77.7 and 98.1.

The control sample shows strong ^{13}C resonances corresponding to the spectrum of mannitol, with smaller resonances for all other compounds. Cells grown in 0.5 M-NaCl show enhanced resonances at δ 63.8 and 73.2, due to glycerol/erythritol. In contrast, cells grown in 0.5 M- CuSO_4 show mannitol and glycerol/erythritol resonances of comparable magnitude. Little evidence of change in the other compounds was evident from the results in Fig. 1.

Cell water and amounts of osmotically-important intracellular solutes in *P. ochro-chloron*, after growth in various Na^+ or Cu^{2+} concentrations, are shown in Table 1. The same compounds, as qualitatively determined by ^{13}C NMR, were also identified by GLC but this latter method enabled quantification. However, GLC does not discriminate between trimethylsilyl derivatives of mannitol and sorbitol (Holligan & Drew, 1971), so the values in Table 1 are termed 'hexitol', but mannitol will predominate as it is found in substantially greater amounts than sorbitol (Fig. 1). On a dry weight basis, glycerol, erythritol and trehalose all showed increases (arabitol only in Na^+) with higher ionic concentration whereas 'hexitol' concentrations were approximately constant. Glycerol showed the greatest relative increase with values 7.8 and 8.7 times greater than the control for 0.5 M- Cu^{2+} and Na^+ , respectively. When account was taken of cell water, which showed a general decrease to about 55% of the control value at 0.5 M- Na^+ or Cu^{2+} (Table 1), glycerol concentrations showed an approximately 15-fold increase relative to the control, for both Cu^{2+} and Na^+ at 0.5 M (Fig. 2). Concentrations of the other low molecular weight organic compounds, in terms of cell water, can be calculated

Table 1. Cell water and organic solute levels ($\pm$ SE) in *P. ochro-chloron* after 2 weeks growth in various concentrations of NaCl or CuSO₄

	Cell water [μ l (mg dry wt) ⁻¹]	Organic solutes [mg (g dry wt) ⁻¹]				
		Glycerol	Erythritol	Arabitol	'Hexitol'	Trehalose
Control	1.26 $\pm$ 0.14	0.88 $\pm$ 0.03	4.92 $\pm$ 0.17	1.14 $\pm$ 0.03	17.98 $\pm$ 0.91	1.83 $\pm$ 0.08
0.05 M-Na ⁺	1.05 $\pm$ 0.11	1.77 $\pm$ 0.02	6.71 $\pm$ 0.13	1.26 $\pm$ 0.04	14.26 $\pm$ 1.32	0.94 $\pm$ 0.11
0.10 M-Na ⁺	0.96 $\pm$ 0.06	3.63 $\pm$ 0.15	8.82 $\pm$ 0.21	1.97 $\pm$ 0.07	16.61 $\pm$ 0.21	1.90 $\pm$ 0.30
0.25 M-Na ⁺	0.81 $\pm$ 0.12	5.78 $\pm$ 0.25	7.84 $\pm$ 0.35	2.34 $\pm$ 0.20	18.11 $\pm$ 1.15	2.93 $\pm$ 0.41
0.50 M-Na ⁺	0.72 $\pm$ 0.07	7.64 $\pm$ 0.79	8.59 $\pm$ 0.06	2.97 $\pm$ 0.02	16.75 $\pm$ 0.84	3.16 $\pm$ 0.14
0.05 M-Cu ²⁺	0.98 $\pm$ 0.07	3.01 $\pm$ 0.21	2.93 $\pm$ 0.42	0.46 $\pm$ 0.09	15.80 $\pm$ 1.20	2.32 $\pm$ 0.15
0.10 M-Cu ²⁺	0.90 $\pm$ 0.10	4.46 $\pm$ 0.26	2.98 $\pm$ 0.18	0.47 $\pm$ 0.02	15.30 $\pm$ 0.49	2.86 $\pm$ 0.11
0.25 M-Cu ²⁺	0.67 $\pm$ 0.07	5.92 $\pm$ 0.31	2.24 $\pm$ 0.16	0.53 $\pm$ 0.12	16.41 $\pm$ 0.37	3.26 $\pm$ 0.44
0.50 M-Cu ²⁺	0.68 $\pm$ 0.09	6.88 $\pm$ 0.25	3.19 $\pm$ 0.11	0.54 $\pm$ 0.06	16.96 $\pm$ 1.15	3.41 $\pm$ 0.25

Fig. 2. Glycerol concentrations in 2-week-old mycelium of *P. ochro-chloron* grown in various concentrations of (a) NaCl and (b) CuSO₄. Bars indicate SE.

from the data in Table 1 but none increased as much as glycerol and only 'hexitol' (1.6 times the relative increase over the control) was present at concentrations similar to or slightly greater than those for glycerol in both Cu²⁺ and Na⁺.

Mycelial yields and cellular levels of Cu²⁺, K⁺ and Na⁺, on a dry weight basis, are shown in Table 2. There was a decrease in biomass with increasing Na⁺, an approximately 50% reduction occurring in 0.5 M-NaCl, but in copper the decrease was not so marked, with only a 20% reduction at 0.5 M-CuSO₄. There was some increase in cell K⁺ at 0.1 and 0.5 M-NaCl, but in the presence of copper there was a uniform decrease to about 74% of the control K⁺ level at all concentrations used. Cell Cu²⁺ was low in control cells and there was no significant difference between values obtained for cells grown in 0.25 and 0.5 M-CuSO₄. Na⁺ was low in all samples although there was some increase with higher external NaCl concentrations. Calculated intracellular osmolalities, assuming Cl⁻ acts as the counterion to all cations present, were generally equivalent to the extracellular osmolality at all but the highest concentrations of Na⁺ and Cu²⁺ (Table 3).

Table 2. Mycelial yields and levels of K^+ , Cu^{2+} and Na^+ ($\pm$ SE) in *P. ochro-chloron* after 2 weeks growth in various concentrations of NaCl or $CuSO_4$

	Mycelium (mg dry wt ml ⁻¹)	Concn [nmol (mg dry wt) ⁻¹]		
		Cell K^+	Cell Cu^{2+}	Cell Na^+
Control	8.73 $\pm$ 0.84	62.64 $\pm$ 2.59	0.33 $\pm$ 0.09	2.40 $\pm$ 0.10
0.10 M-Na ⁺	6.83 $\pm$ 0.48	71.28 $\pm$ 2.38	ND	3.28 $\pm$ 0.82
0.25 M-Na ⁺	5.83 $\pm$ 0.52	60.38 $\pm$ 1.51	ND	4.78 $\pm$ 0.76
0.50 M-Na ⁺	4.27 $\pm$ 0.39	97.66 $\pm$ 1.02	ND	6.51 $\pm$ 0.69
0.10 M- Cu^{2+}	8.44 $\pm$ 0.23	45.14 $\pm$ 3.02	4.80 $\pm$ 0.70	4.92 $\pm$ 2.10
0.25 M- Cu^{2+}	7.73 $\pm$ 0.59	46.02 $\pm$ 0.87	23.20 $\pm$ 2.54	ND
0.50 M- Cu^{2+}	6.73 $\pm$ 0.50	47.50 $\pm$ 0.86	25.37 $\pm$ 1.36	9.24 $\pm$ 3.00

ND, Not determined.

Table 3. Intra- and extracellular osmolalities of *P. ochro-chloron* after 2 weeks growth in various concentrations of NaCl or $CuSO_4$

	Extracellular osmolality [osmol (kg cell water) ⁻¹]	Intracellular osmolality* [osmol (kg cell water) ⁻¹]
Control	0.22	0.23
0.10 M-Na ⁺	0.40	0.38
0.25 M-Na ⁺	0.68	0.47
0.50 M-Na ⁺	1.14	0.67
0.10 M- Cu^{2+}	0.33	0.32
0.25 M- Cu^{2+}	0.47	0.50
0.50 M- Cu^{2+}	0.68	0.55

* Calculated from data in Tables 1 and 2, assuming Cl^- acts as counterion.

DISCUSSION

In this study the marked copper tolerance of *P. ochro-chloron* has been confirmed as has its ability to grow in solutions of high salinity. Fungi that grow in saline conditions often accumulate one or more intracellular solutes, including polyols and/or low molecular weight saccharides, which help to maintain osmotic balance and generate the positive turgor pressures required for growth (see Jennings, 1983) and also confer protection against NaCl inactivation of enzymes (see Borowitzka & Brown, 1974; Brown, 1983). The major compounds present in *P. ochro-chloron* were glycerol, mannitol, sorbitol, arabitol, erythritol and trehalose, with glycerol showing the greatest response to increasing external ionic concentration (equivalent to decreasing water activity). High induced glycerol concentrations, as a response to increased salinity, have been frequently reported for yeasts including *Saccharomyces rouxii* and *S. cerevisiae*, *Debaromyces hansenii*, *Pichia miso* and filamentous fungi including *Dendryphiella salina*, *Penicillium chrysogenum* and *Chaetomium globosum* (see Jennings, 1983). The ecology of *P. ochro-chloron* is poorly understood and there are, to our knowledge, no reports of its occurrence in marine habitats, but this glycerol-producing ability is not confined to marine or halotolerant fungi (Brown, 1978). Copper caused almost identical marked changes in glycerol concentrations within cells, which agrees with the hypothesis that glycerol production is a direct response to a decrease in water activity of the medium (Brown, 1976, 1978).

Sodium is toxic at high concentrations in marine fungi (Jones & Byrne, 1976; Jennings, 1983); *P. ochro-chloron* contained very low amounts of sodium. The osmolality of 0.5 M-NaCl is approximately twice that of 0.5 M- $CuSO_4$ (Table 3) yet glycerol concentrations were approximately equal in cells grown in equal concentrations of both solutes. However, the increases in K^+ and erythritol that occurred with higher external NaCl concentrations but not with copper (see Tables 1 and 2) may account for the apparent shortfall in intracellular glycerol concentration in Na^+ -containing medium. The uniform decrease in cell K^+ with

increasing Cu^{2+} concentration may indicate some membrane disruption, but since there was only a slight decrease in yield at the highest Cu^{2+} concentration, this implies that K^+ release may have occurred to compensate for Cu^{2+} uptake. Stoichiometric release of K^+ in conjunction with heavy metal uptake is found in several yeasts and fungi at non-toxic concentrations (Norris & Kelly, 1977; Mowll & Gadd, 1984). However, when values are converted to unit cell water, by means of the data in Table 1, the losses are generally insignificant. Changes in intracellular solute content are sufficient to balance the increases in extracellular osmolality at all but the highest concentrations of Na^+ and Cu^{2+} . The apparent shortfall at the highest osmolalities, if experimental variation is insignificant, could be due to the presence of unidentified intracellular inorganic solutes.

Total cell copper values were low, especially in view of the high external concentrations, and the observation that a saturation value was reached implies some control of cellular uptake. This constant copper content has also been noted previously (Okamoto & Fuwa, 1974; Okamoto *et al.*, 1977) with values of about 16 nmol mg^{-1} at copper concentrations from 16 mM up to saturation values. There is wide variation in other reported values, e.g. Baldry & Dean (1980) found 32 nmol mg^{-1} taken up from 0.08 mM ; Basu *et al.* (1955) obtained a maximum of 630 nmol mg^{-1} from 50 mM and Stokes & Lindsay (1979) obtained $6300 \text{ nmol mg}^{-1}$ after 1 d in solutions of 8, 16 and 80 mM (figures calculated from cited references). While some variation may be due to strain and cultural differences, it is likely that inadequate washing of mycelium, always a problem with filamentous fungi, may have led to some of the higher estimations. There are few published values of copper accumulation for other filamentous fungi.

While low pH can reduce heavy metal toxicity to fungi by reducing uptake (Starkey, 1973; Gadd & Griffiths, 1980) and may have had some contribution in this study, the huge excess of Cu^{2+} and Na^+ present still implies some cellular control of influx. From this study, we conclude that the high concentrations of cellular glycerol, induced by high Cu^{2+} and Na^+ , have a significant osmotic effect, allowing growth in media of high osmotic strength. The exclusion of Cu^{2+} may be considered as a gratuitous resistance mechanism (Gadd & Griffiths, 1978) since it is not specific for copper and can also apply to sodium. However, such ion exclusion and intracellular osmotic compensation involving polyols is of clear ecological and physiological significance for organisms which grow in marine and/or low water activity habitats.

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