Zinc Uptake and Toxicity in the Yeasts *Sporobolomyces roseus* and *Saccharomyces cerevisiae*

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Sporobolomyces roseus and *Saccharomyces cerevisiae* accumulated zinc from zinc-containing medium. Uptake was biphasic and consisted of an initial, rapid, metabolism-independent binding of zinc to cell surfaces which was followed by slower, metabolism-dependent intracellular uptake of zinc. *Spor. roseus* could bind approximately eight times more zinc, per unit surface area, than could *S. cerevisiae*. Metabolism-dependent zinc uptake followed Michaelis-Menten kinetics with K_m values of 0.09 and 5.00 mM- Zn^{2+} for *Spor. roseus* and *S. cerevisiae*, respectively; corresponding V_{max} values were 0.51 and 9.09 nmol Zn^{2+} (mg dry wt) $^{-1}$ min $^{-1}$. Zinc uptake by viable cells was not accompanied by potassium release in either yeast, but zinc levels which affected viability in *S. cerevisiae* caused this yeast to release K^+ . No efflux of K^+ was observed for *Spor. roseus* despite its greater sensitivity to zinc.

INTRODUCTION

Zinc is a metal generally considered to be low in toxicity to fungi and yeasts (Somers, 1961; Ross, 1975). *Neocosmospora vasinfecta* and *Candida albicans* could grow in zinc concentrations of up to 0.1 and 0.3 mM, respectively (Paton & Budd, 1972; Ross, 1982), while *Candida utilis* accumulated high levels of intracellular zinc [up to 122 nmol Zn^{2+} (mg dry wt) $^{-1}$] without any reduction of growth rate or viability (Failla & Weinberg, 1977; Lawford *et al.*, 1980).

Cation uptake by yeasts is typically biphasic, involving rapid, metabolism-independent binding to cell surfaces, followed by a slower phase of metabolism-dependent intracellular uptake (Rothstein *et al.*, 1958; Rothstein, 1959; Passow & Rothstein, 1960; Fuhrmann & Rothstein, 1968; Ponta & Broda, 1970; Norris & Kelly, 1977; Failla *et al.*, 1976; Failla & Weinberg, 1977; Gadd & Mowll, 1983). The uptake of metal cations by *Saccharomyces cerevisiae* is generally accompanied by potassium efflux which may be an integral part of the physiological mechanism for maintenance of the ionic balance or may be a symptom of membrane disruption and cell death (Passow & Rothstein, 1960; Norris & Kelly, 1977; Gadd & Mowll, 1983). Although metal uptake can be related to toxicity in some organisms – resistant strains take up less metal than do sensitive strains (Chopra, 1971; Gadd & Griffiths, 1978, 1980) – the relationship between uptake and potassium efflux is not clear, mainly because of the lack of attention paid to viability loss (Gadd & Mowll, 1983).

The objectives of this study were to ascertain the relationship between zinc uptake, potassium efflux and viability loss in *Saccharomyces cerevisiae* and in *Sporobolomyces roseus* Kluyver *et van Niel*, an organism extremely sensitive to heavy metal ions (Bewley & Campbell, 1980; Gadd, 1983).

METHODS

Organisms, media and growth conditions. *Saccharomyces cerevisiae* (NCYC 78) and *Sporobolomyces roseus*, originally obtained from Dr C. Edwards, Department of Microbiology, University of Liverpool, were used. For routine maintenance, *S. cerevisiae* was grown on MYGP medium of composition (g l $^{-1}$): malt extract (Lab-M), 3.0; yeast extract (Lab-M), 3.0; bacteriological peptone (Oxoid), 5.0; D-glucose, 10.0; agar (Lab-M, grade 2), 12.0. *Spor. roseus* was grown on the following medium (g l $^{-1}$): bacteriological peptone (Oxoid), 3.0; yeast extract (Lab-

M), 5.0; casein hydrolysate (BDH), 5.0; D-glucose, 40.0; agar (Lab-M, grade 2), 12.0. For liquid cultures of both organisms the defined medium of Norris & Kelly (1977) was used with added trace elements ($\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.004 g l^{-1} ; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004 g l^{-1} ; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0004 g l^{-1}). Starter cultures were prepared by loop inoculating 50 ml culture medium which was incubated for 24 h. These were used to inoculate experimental flasks, containing 100 ml medium, to an initial cell density of $5 \times 10^6 \text{ ml}^{-1}$. Liquid cultures were incubated at 25°C on an orbital shaker (100 r.p.m.).

Preparation of cell suspensions. Cells were harvested in the exponential phase of growth ($1\text{--}2 \text{ mg dry wt ml}^{-1}$) by centrifugation ($2000 \text{ g} \times 5 \text{ min}$), washed twice in and finally resuspended in 5 mM-PIPES buffer, pH 6.5, to give a dry weight of approximately 0.4 mg ml^{-1} . Suspensions were incubated at 25°C on a magnetic stirrer for 60 min prior to the addition of glucose (final concentration 50 mM) or distilled water. After a further 60 min, appropriate amounts of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ were added to the suspensions. Where required, the endogenous respiration inhibitor, antimycin A (Sigma) was added (final concentration $160 \mu\text{M}$) 30 min before the zinc addition.

Ion analysis. Zinc uptake was followed by the inclusion of ^{65}Zn (Amersham) in the stocks of cold $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ so that on addition to cell suspensions activities of $0.1\text{--}0.3 \mu\text{Ci ml}^{-1}$ ($3.7\text{--}11.1 \text{ KBq ml}^{-1}$) were obtained. Samples of the cell suspensions (0.8 ml) were taken at intervals and the cells separated from the medium by microcentrifugation through 0.5 ml of a 'dinonyl' phthalate/silicone fluid mixture (3:2, v/v) into 0.2 ml of a lethal solution of 40% (v/v) perchloric acid (Werden *et al.*, 1972). After removal of the medium, samples of the perchloric acid layer were removed and counted in scintillation fluid based on toluene and Triton X-100 (2:1, v/v) and containing 4.0 g PPO l^{-1} and $0.1 \text{ g POPOP l}^{-1}$, using a Packard 300 CD scintillation counter. Potassium release was followed by periodic removal of 5 ml samples which were centrifuged ($2000 \text{ g} \times 5 \text{ min}$). Pellets were washed with distilled water, recentrifuged, extracted with 6 M-nitric acid (100°C , 30 min) and analysed for potassium using an EEL flame photometer.

Other methods. Viability was assessed by the conventional spread plate technique using distilled water as diluent. Dry weights were determined using tared aluminium foil cups dried to constant weight at 105°C . Surface areas of the cell suspensions were estimated by calculation following microscopical measurement of the cells using an eyepiece graticule. Cells of *Spor. roseus* were assumed to be cylindrical with hemispherical ends, while cells of *S. cerevisiae* were assumed to be spherical.

Chemicals. All chemicals used were of analytical grade. 'Dinonyl' phthalate [bis (3,5,5-trimethylhexyl) phthalate] (Fluka) was obtained from Fluorochem Ltd, Glossop, Derbyshire, U.K. and Dow Corning 550 silicone fluid from Hopkin & Williams.

RESULTS

Biphasic accumulation of zinc from zinc-containing medium occurred in both yeasts. The initial uptake was rapid and independent of glucose, antimycin A or temperature (Fig. 1) and corresponded to the binding of Zn^{2+} ions to sites on the cell surface. At a zinc concentration of 0.1 mM, surface binding was estimated at 28.9 and $3.8 \text{ nmol (mg dry wt)}^{-1}$ for *Spor. roseus* and *S. cerevisiae*, respectively. When expressed in terms of surface area, corresponding values for the two organisms were 10.8 and 1.1 nmol mm^{-2} (Fig. 1). In both yeasts, initial adsorption values at a wide range of zinc concentrations (0.01–0.5 mM) gave a straight line when plotted according to the Freundlich equation (Freundlich, 1926) and a mass/law plot (Scatchard, 1949) of such values gave the dissociation constant (K_D) and the number (Y_i) of high affinity binding sites from the reciprocal of the steeper slope and its intercept, respectively (Fig. 2). Calculated K_D values were $4.6 \mu\text{M-Zn}^{2+}$ for *Spor. roseus* and $9.8 \mu\text{M-Zn}^{2+}$ for *S. cerevisiae*, the lower value of the former corresponding with the higher affinity for zinc. The Y_i values were 14.8 and $4.7 \text{ nmol Zn}^{2+} \text{ (mg dry wt)}^{-1}$ for *Spor. roseus* and *S. cerevisiae*, respectively, again emphasizing the higher surface binding capacity for zinc of *Spor. roseus*.

The amounts of surface-bound zinc were subsequently exceeded by amounts of zinc taken up into the cells (Fig. 1), a process dependent on the presence of glucose. There was little second phase uptake of zinc in the presence of antimycin A or at 4°C . At a concentration of 0.1 mM, *S. cerevisiae* took up considerably more zinc than *Spor. roseus*, initial uptake rates being 2.52 and $0.40 \text{ nmol (mg dry wt)}^{-1} \text{ min}^{-1}$, respectively. This active accumulation followed Michaelis-Menten kinetics in both organisms and measurement of initial uptake rates at a range of zinc concentrations (0.01–0.5 mM) gave K_m values of 0.09 ± 0.01 and $5.00 \pm 0.41 \text{ mM-Zn}$ for *Spor. roseus* and *S. cerevisiae*, respectively; corresponding V_{max} values were 0.51 ± 0.05 and $9.09 \pm 0.85 \text{ nmol Zn (mg dry wt)}^{-1} \text{ min}^{-1}$.

Fig. 1. Zn^{2+} accumulation by (a) *Spor. roseus* and (b) *S. cerevisiae* from 0.1 mM- ZnSO_4 . At 25 °C in the presence (○) or absence (●) of glucose; in the absence of glucose and the presence of 160 μM -antimycin A (▲); at 4 °C in the presence of glucose (■). The results shown are the means of three experiments.

Fig. 2

Fig. 3

Fig. 2. Mass/low plot of surface binding of Zn^{2+} from a range of ZnSO_4 concentrations for *Spor. roseus* (●) and *S. cerevisiae* (○). The reciprocal of the steeper slope gave the dissociation constant (K_D) while the intercept on the abscissa gave the capacity (Y_i) of the high affinity Zn^{2+} binding sites. Standard errors are indicated by horizontal lines through points.

Fig. 3. (a) Effect of zinc on cell K^+ levels in *Spor. roseus* (●) and *S. cerevisiae* (○). Control cell levels were 150 and 290 nmol K^+ (mg dry wt) $^{-1}$, respectively. (b) Effect of zinc on viability of *Spor. roseus* (●) and *S. cerevisiae* (○). Cell K^+ and viability were measured after 3 h incubation and standard errors are indicated by vertical lines through points.

Accumulation of zinc by viable cells was not accompanied by any measurable extracellular release of K^+ ions from either organism. However, at concentrations of zinc that induced viability loss in *S. cerevisiae* (>0.1 mM), K^+ release was evident (Fig. 3).

Spor. roseus was much more sensitive at lower concentrations of zinc than *S. cerevisiae* and there was a general decrease in viability of the cells with increasing zinc concentrations. This was in contrast to the rapid loss of viability of *S. cerevisiae* above a threshold level of 0.1 mM-zinc (Fig. 3). However, despite the apparent greater sensitivity of *Spor. roseus* to zinc, there was no measurable K^+ release (Fig. 3). In both yeasts the loss of viability was irreversible.

DISCUSSION

Differences in the surface binding capacities of each yeast were probably due to differences in cell wall structure. Crook & Johnston (1962) found that while the walls of *S. cerevisiae* were composed of glucan and mannan with traces of chitin, the walls of *Spor. roseus* consisted of chitin and mannan with little glucan and, in addition, small amounts of γ -aminobutyric acid. The red pigmentation of the walls of *Pyrenophora avenae* removed phenyl mercuric ions from a solution of phenyl mercuric acetate (Greenaway, 1971) and it is possible that the red pigment of *Spor. roseus*, although different in composition to that of *P. avenae*, was similarly involved in zinc binding.

The uptake of zinc in non-lethal amounts by *Spor. roseus* and *S. cerevisiae* took place without any exchange of intracellular K^+ which contrasted with the uptake of other heavy metals by yeasts (Passow & Rothstein, 1960; Norris & Kelly, 1977; Gadd & Mowll, 1983). However, many previous studies on metal uptake have not considered viability and it is often unclear whether K^+ release is an integral part of an uptake system or merely represents membrane disruption. In *S. cerevisiae*, while cadmium may be taken up by a glucose-dependent system at low concentrations, K^+ release was due to membrane damage and cell death (Gadd & Mowll, 1983). Passow & Rothstein (1960) suggested that the increased K^+ release from yeast exposed to increasing mercury levels was due to more cells showing an all-or-nothing response and there appeared to be a similar relationship in this study.

Although the relationship between zinc uptake, K^+ release and toxicity appeared relatively straightforward with *S. cerevisiae*, *Spor. roseus* exhibited several unusual features. Although much more sensitive to zinc than *S. cerevisiae* (see also Gadd, 1983), it took up considerably less zinc into the cells and there was no loss of intracellular K^+ despite massive viability loss. In some organisms it has been found that sensitivity to a metal is related to amounts accumulated (Chopra, 1971; Tynecka *et al.*, 1975; Gadd & Griffiths, 1978, 1980). The large amounts of zinc taken up by *S. cerevisiae* were indeed related to toxicity, i.e. extensive K^+ release and irreversible viability loss. It is clear that a different mechanism of zinc toxicity occurred in *Spor. roseus*. There appeared to be no membrane disruption and so the zinc may have exerted toxic action by denaturation of enzymes or other proteins within the cytoplasm.

This study illustrates the connection between zinc uptake, K^+ release and toxicity in *S. cerevisiae* and also shows that there is a substantially different mechanism of zinc sensitivity in *Spor. roseus*, uptake and toxicity occurring with no concomitant K^+ release.

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