Cadmium Uptake by *Aureobasidium pullulans*

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(Received 1 August 1983)

Yeast-like cells, mycelium and melanin-pigmented chlamydozoospores of *Aureobasidium pullulans* accumulated cadmium from cadmium-containing medium. With chlamydozoospores, uptake was rapid and independent of temperature and the presence of glucose, and corresponded to binding of cadmium to cell surfaces. With yeast-like cells and mycelium, the initial rapid surface adsorption of cadmium was up to six times lower than that of chlamydozoospores, and was followed by metabolism-dependent transport of cadmium into the cytoplasm. The second phase of uptake was slower and followed Michaelis–Menten kinetics. Concomitant with the intracellular accumulation of cadmium was an efflux of potassium, two K^+ ions being released for each Cd^{2+} ion accumulated. Potassium loss was low in comparison to the cell potassium levels and there was no loss of viability by any cell type at cadmium concentrations up to 0.5 mM. In yeast-like cells and mycelium, Ca^{2+} ions were found to inhibit cadmium uptake competitively. Work with the ionophore valinomycin confirmed that potassium efflux and cadmium influx occurred at different sites on the plasma membrane of yeast-like cells. Intracellular cadmium could be actively excreted from cells.

INTRODUCTION

Cation uptake by yeasts and fungi is usually biphasic, involving an initial rapid metabolism-independent uptake corresponding to the binding of the cations to negatively charged sites in the cell wall, followed by slower, metabolism-dependent uptake into the cytoplasm (see for example Rothstein *et al.*, 1958; Failla *et al.*, 1976). In yeasts, cation uptake can be accompanied by an efflux of potassium (Norris & Kelly, 1977) which may be a physiological mechanism for the maintenance of ionic balance across the membrane. However, potassium efflux may also be symptomatic of membrane disruption and cell death; this has clearly been shown with *Saccharomyces cerevisiae* for zinc (Mowll & Gadd, 1983) and cadmium (Gadd & Mowll, 1983). The relationship between heavy metal uptake, potassium release and viability in fungi other than yeasts has received little attention.

Aureobasidium pullulans (de Bary) Arnaud is a polymorphic fungus whose morphogenetic transitions involving yeast-like cells, hyphae and chlamydozoospores have received some attention (see for example Ramos & Garcia Acha, 1975; Dominguez *et al.*, 1978). It exhibits considerable tolerance towards heavy metals (Smith, 1977; Gadd, 1983) and is frequently isolated from phylloplanes contaminated with cadmium, lead and zinc where it may be the dominant organism (Smith, 1977; Bewley & Campbell, 1978, 1980). Like many other potentially toxic metals, cadmium can induce or accelerate the formation of melanin-pigmented structures in *A. pullulans* (Gadd, 1981) but there is little information on physiological responses to this metal.

This paper describes the specificity and kinetics of cadmium uptake, in relation to toxicity, by different cell types of *A. pullulans*.

Abbreviations: DDG, 2-deoxy-D-glucose; NTA, nitrilotriacetic acid.

METHODS

Organism, media and growth conditions. *Aureobasidium pullulans* (IMI 45533) was maintained on MYGP agar of composition (g l^{-1}): malt extract (Lab-M), 3; yeast extract (Lab-M), 3; bacteriological peptone (Oxoid), 5; D-glucose, 10; agar (Lab-M, grade 2), 12. Liquid cultures were grown in a medium, pH 6.8, of composition (g l^{-1}): KH_2PO_4 , 2.72; K_2HPO_4 , 5.22; $(\text{NH}_4)_2\text{SO}_4$, 1.98; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.12; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0022; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.004; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0004; D-glucose, 20. Starter cultures were prepared by loop-inoculating 50 ml of 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 $5 \times 10^6 \text{ ml}^{-1}$. All liquid cultures were incubated at 25°C on an orbital shaker (100 cycles min^{-1}).

Preparation of cell suspensions. Yeast-like cells were harvested in the exponential phase of growth (1–2 mg dry wt ml^{-1}). Chlamydospores were obtained by settling late stationary phase cultures (> 20 d old) for approximately 30 min and subsequently removing the supernatant and top layer of the pellet, which were discarded. Cells were resuspended in distilled water and the process repeated until the preparation was at least 95% pure. Mycelium was obtained from cultures grown in the liquid medium with added 0.5% (w/v) yeast extract (Difco) for 12 h, by filtration through a $63 \mu\text{m}$ aperture mesh. All cell preparations were centrifuged at 2000 g for 5 min, and washed twice with and resuspended in 5 mM-PIPES buffer, pH 6.5, to give a dry weight of approximately 0.4 mg ml^{-1} . In experiments on the effect of pH on cadmium uptake, phthalate buffer was used. Use of a Cd^{2+} -specific electrode (Orion Research, Inc., 380 Putnam Avenue, Cambridge, MA 02139, USA) revealed that, for 0.1 mM- Cd^{2+} in phthalate buffer, the availability of free Cd^{2+} was decreased by approximately 30% for all pH values used. Suspensions were incubated at 25°C on a magnetic stirrer for 30 min prior to the addition of glucose (final concentration 50 mM) or distilled water. After a further 30 min, appropriate amounts of $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ were added to the suspensions. Where required, the following were added 10 min before the cadmium: potassium cyanide (final concentration 0.2 mM); 2-deoxy-D-glucose (DDG) (Sigma) (final concentration 50 mM); valinomycin (Sigma) (final concentration 18 μM); and Dio-9 (final concentration 0.02 mg ml^{-1}).

Ion uptake and analysis. Cadmium uptake was followed by the incorporation of ^{109}Cd (Amersham) in the stock solutions of $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ so that on addition to cell suspensions activities of 0.1–0.3 $\mu\text{Ci ml}^{-1}$ (3.7–11.1 kBq ml^{-1}) were obtained. Samples of cell suspensions (0.8 ml) were taken at intervals and the cells separated from the medium by centrifugation through 0.5 ml of a 'dinonyl' phthalate/silicone fluid mixture (3:2, v/v) into 0.2 ml of 40% (v/v) perchloric acid (Werden *et al.*, 1972). After removal of the medium, samples of the perchloric acid layer were counted in a scintillation fluid based on toluene and Triton X-100 (2:1, v/v) and containing 2,5-diphenyloxazole (4 g l^{-1}) and 1,4-di-2-(5-phenyloxazolyl)benzene (0.1 g l^{-1}), using a Packard 300 CD scintillation counter. To study cadmium efflux, yeast-like cell suspensions were prepared aseptically and incubated in sterile PIPES buffer with labelled 0.1 mM- CdSO_4 and glucose. After 12 h the cells were washed with and resuspended in PIPES buffer, with or without 50 mM-glucose, and samples were taken as described above. Potassium release was measured as previously described (Gadd & Mowll, 1983) using an EEI 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 yeast-like cells and chlamydospores were calculated following microscopical measurement using an eyepiece graticule. Yeast-like cells were assumed to be cylindrical with hemispherical ends, while chlamydospores were assumed to consist of two slightly overlapping spheres.

Chemicals. All chemicals used were of analytical grade. Dio-9 was a kind donation from Gist-Brocades NV, Delft, Holland. 'Dinonyl' phthalate [bis(3,5,5-trimethylhexyl)phthalate] (Fluka) was obtained from Fluorochem, Glossop, Derbyshire, UK and Dow Corning 550 silicone fluid from Hopkin and Williams.

RESULTS

Chlamydospores differed considerably from yeast-like cells and mycelium with regard to cadmium uptake (Table 1). While the latter two cell types displayed biphasic cation uptake characteristics which involved rapid, metabolism-independent binding of cadmium to cell surfaces followed by metabolism-dependent uptake of cadmium into the cytoplasm, chlamydospores showed only the first phase, that of surface binding. In all cell types, surface adsorption of cadmium was not dependent on glucose and temperature and, for a range of cadmium concentrations (0.01–1.00 mM), initial adsorption values gave straight lines when plotted according to the Freundlich isotherm (Freundlich, 1926). However, chlamydospores were able to adsorb much larger quantities of cadmium to surfaces than yeast-like cells or mycelium and this was not due to differences in surface area of the two cell types. A mass/law plot (Scatchard, 1949) of cadmium adsorbed gave the capacity (Y_1) and the dissociation constant (K_D) of the high-affinity cadmium-binding sites (Table 2). The Y_1 values emphasized the greater

Table 1. *Surface binding and cadmium influx to different cell types*

The units for surface binding are nmol (mg dry wt)⁻¹ and those for cadmium influx pmol (mg dry wt)⁻¹ min⁻¹. Figures in parentheses were calculated with respect to surface area (units nmol mm⁻² and pmol mm⁻² min⁻¹, respectively). The results are shown $\pm$ SE.

Cd ²⁺ concn (mM)	Yeast-like cells		Mycelium		Chlamydo spores	
	Surface binding	Cd ²⁺ influx	Surface binding	Cd ²⁺ influx	Surface binding	Cd ²⁺ influx
0.01	3.5 $\pm$ 0.1 (1.09 $\pm$ 0.03)	11.0 $\pm$ 4.0 (3.4 $\pm$ 1.2)	1.1 $\pm$ 0.2	22.8 $\pm$ 5.0	4.8 $\pm$ 1.3 (3.8 $\pm$ 1.0)	0.0
0.05	7.9 $\pm$ 0.7 (2.45 $\pm$ 0.22)	43.4 $\pm$ 7.0 (13.5 $\pm$ 2.2)	3.1 $\pm$ 0.4	38.9 $\pm$ 6.4	14.7 $\pm$ 1.7 (11.8 $\pm$ 1.4)	0.0
0.10	10.0 $\pm$ 0.3 (3.10 $\pm$ 0.09)	83.9 $\pm$ 11.6 (26.0 $\pm$ 3.6)	8.5 $\pm$ 1.0	62.6 $\pm$ 8.8	24.6 $\pm$ 2.7 (19.7 $\pm$ 2.2)	0.0
0.50	19.1 $\pm$ 1.0 (5.92 $\pm$ 3.1)	105.8 $\pm$ 15.3 (32.8 $\pm$ 4.7)	22.0 $\pm$ 2.4	114.7 $\pm$ 12.4	37.0 $\pm$ 1.5 (29.6 $\pm$ 1.2)	0.0
1.00	27.4 $\pm$ 3.8 (8.50 $\pm$ 1.18)	122.3 $\pm$ 16.2 (37.9 $\pm$ 5.0)	44.0 $\pm$ 3.3	130.6 $\pm$ 15.2	66.3 $\pm$ 4.2 (53.0 $\pm$ 3.4)	0.0

Table 2. *Kinetics of cadmium uptake and potassium release by different cell types*

Cell type	Cd ²⁺ binding		Cd ²⁺ influx		K ⁺ release	
	K _D (μ M)	Y _t (nmol mg ⁻¹)	K _m (mM)	V _{max} (nmol mg ⁻¹ min ⁻¹)	K _m (mM)	V _{max} (nmol mg ⁻¹ min ⁻¹)
Yeast-like cells	24	12	0.11	0.15	0.09	0.30
Mycelium	29	4	0.10	0.15	0.10	1.00
Chlamydo spores	75	42	—	—	—	—
Yeast-like cells + Ca ²⁺	35	11	0.24	0.12	ND	ND
Mycelium + Ca ²⁺	37	3	0.18	0.11	ND	ND

ND, Not determined.

Table 3. *Uptake of cadmium by yeast-like cells from 0.1 mM-CdSO₄ solution under different external conditions*

The results are shown $\pm$ SE.

Treatment	Surface binding (nmol (mg dry wt) ⁻¹)	Cd ²⁺ influx (pmol (mg dry wt) ⁻¹ min ⁻¹)
Control	10.0 $\pm$ 0.3	83.9 $\pm$ 11.6
Without glucose	11.1 $\pm$ 1.4	0.3 $\pm$ 0.2
With 50 mM-DDG	10.8 $\pm$ 0.9	0.3 $\pm$ 0.2
At 4 °C	9.0 $\pm$ 0.8	31.1 $\pm$ 4.1
With 0.2 mM-KCN	3.0 $\pm$ 0.4	32.2 $\pm$ 3.1

binding capacity of the chlamydo spores. Of the divalent cations tested, Hg²⁺, Co²⁺, Mg²⁺, Mn²⁺ and Cu²⁺ inhibited surface binding of cadmium by yeast-like cells by at least 60%, while Ca²⁺ and Zn²⁺ had no effect. Similar results were noted for mycelium, but with chlamydo spores only Cu²⁺ significantly inhibited surface binding of cadmium.

Intracellular accumulation of cadmium occurred in yeast-like cells and mycelium (Table 1). For yeast-like cells, uptake was dependent on the presence of glucose and was depressed at low temperature (4 °C) and by the presence of DDG [an analogue of glucose which can be assimilated but not used as an energy source (Van Steveninck, 1968)] and potassium cyanide (Table 3). Intracellular uptake was also found to be metabolism-dependent in mycelium (results not shown). This active accumulation followed Michaelis–Menten kinetics and Lineweaver–Burk plots of initial rates of uptake gave the dissociation constant (K_m) and theoretical

Table 4. *Effect of pH on the uptake of cadmium by different cell types*

The control values (100%) for Cd^{2+} binding were $[\text{nmol} (\text{mg dry wt})^{-1}]$: yeast-like cells, 6.3 ± 0.9 ; mycelium, 4.0 ± 0.6 ; chlamydospores, 23.4 ± 3.5 ; those for Cd^{2+} influx were $[\text{pmol} (\text{mg dry wt})^{-1} \text{ min}^{-1}]$: yeast-like cells, 45.3 ± 6.3 ; mycelium, 35.0 ± 5.0 .

pH	Cd^{2+} binding (% of control)			Cd^{2+} influx (% of control)	
	Yeast-like cells	Mycelium	Chlamydospores	Yeast-like cells	Mycelium
6.5	100	100	100	100	100
5.5	78	86	88	84	93
4.5	49	69	47	69	83
3.5	37	70	32	25	23
2.5	12	53	12	21	12

Table 5. *Effect of cadmium on the viability of different cell types*

Viability was estimated after 3 h incubation in the presence of CdSO_4 .

Cd^{2+} concn (mM)	$10^{-6} \times \text{Viable counts (c.f.u. ml}^{-1})$		
	Yeast-like cells	Mycelium	Chlamydospores
0.00	11.8 ± 1.3	2.28 ± 0.08	1.42 ± 0.09
0.01	9.32 ± 0.24	2.42 ± 0.12	ND
0.05	ND	ND	1.33 ± 0.07
0.10	10.7 ± 3.6	2.34 ± 0.04	ND
0.50	8.49 ± 0.29	2.34 ± 0.09	1.36 ± 0.06
1.00	9.77 ± 0.41	1.67 ± 0.12	1.26 ± 0.16

ND, Not determined.

maximum rate of transport (V_{max}) for the two cell types (Table 2). Of cations added in equimolar amounts to the cadmium, only calcium inhibited cadmium transport by yeast-like cells and mycelium and this inhibition was competitive, with little change in the V_{max} and an increase in the K_m , which corresponded to an approximate halving of the cadmium affinity of the transport sites (Table 2). Addition of Zn^{2+} , Co^{2+} , Hg^{2+} , Mg^{2+} and Cu^{2+} had no effect on the rates of intracellular cadmium transport of either cell type. Inclusion of the divalent cation ionophore A23187 (Reed, 1979) in the medium did not increase the rate of cadmium uptake.

A lowered pH of the medium decreased both cadmium adsorption and cadmium transport for all cell types (Table 4).

Concomitant with intracellular uptake of cadmium by yeast-like cells and mycelium was an efflux of potassium into the medium. This efflux was uniphasic and its rate was approximately half that of cadmium influx. It was found for yeast-like cells that the V_{max} of K^+ efflux was twice that of cadmium influx while the K_m values were very similar (Table 2). However, for mycelium the V_{max} of K^+ efflux was 6.7 times greater than the V_{max} of cadmium influx (Table 2). The K^+ -specific ionophores valinomycin (Henderson *et al.*, 1969) and Dio-9 (Boutry *et al.*, 1977) increased potassium release by a factor of approximately 1.6 but this did not cause an increase in cadmium influx. There was a negligible efflux of potassium from chlamydospores. In all cell types, potassium release was not accompanied by any significant loss in viability at cadmium concentrations up to 0.5 mM and over the 3 h experimental period (Table 5).

When yeast-like cells exposed to cadmium were washed four times with distilled water, 27.6% of the total cellular cadmium was removed; washing with NTA removed 98% of the total cellular cadmium. In addition, there was an efflux of cadmium from cadmium-loaded yeast-like cells, the rate of which was $31.7 \pm 0.7 \text{ pmol} (\text{mg dry wt})^{-1} \text{ min}^{-1}$, but when glucose was added (final concentration 50 mM), the rate of cadmium efflux was increased to $114.8 \pm 0.8 \text{ pmol} (\text{mg dry wt})^{-1} \text{ min}^{-1}$.

DISCUSSION

Differences in cell wall structure accounted for the considerable differences in the amount of cadmium bound by chlamydo-spores and the other cell types. The greater thickness of the chlamydo-spore wall probably accounted for some of the greater binding capacity, and it is possible that the melanin component present only in chlamydo-spore walls was also involved. Natural melanin has been shown to have many binding sites for Cu^{2+} (Sarna *et al.*, 1980) and Bruenger *et al.* (1967) showed that most of the metal ions bound to naturally occurring melanin granules were bound to the melanin constituent and not the protein. In addition, the induction of melanization by copper and cadmium ions has been shown in *A. pullulans* (Gadd & Griffiths, 1980a; Gadd, 1981).

While intracellular transport of cadmium did occur in yeast-like cells and mycelium, it did so at much lower rates than those reported for metal accumulation by yeasts such as *Saccharomyces cerevisiae* which could accumulate over $300 \text{ nmol Cd}^{2+} (\text{mg dry wt})^{-1}$ after 60 min in 0.5 mM CdSO_4 (Norris & Kelly, 1977; Gadd & Mowll, 1983) and *Sporobolomyces roseus* which accumulated $90 \text{ nmol Zn}^{2+} (\text{mg dry wt})^{-1}$ after 180 min in 0.1 mM-ZnSO_4 (Mowll & Gadd, 1983). This low uptake even at high cadmium concentrations was indicative of some control on the uptake system which allowed the cells to maintain their viable state, and it has been reported that a copper-resistant strain of *A. pullulans* takes up less copper than a sensitive strain (Gadd & Griffiths, 1980b). It was evident that calcium competed for the cadmium transport sites and it is therefore likely that cadmium was transported via an existent calcium uptake system. This was probably due to a similarity in ionic radii (0.097 nm for Cd^{2+} and 0.099 nm for Ca^{2+}). Norris & Kelly (1977) also noted the inhibition of Cd^{2+} uptake by Ca^{2+} in *Sacch. cerevisiae*. Other cations tested (except Zn^{2+}) inhibited surface binding of cadmium, but not its transport into the cytoplasm, and so maximum surface binding was not a prerequisite to intracellular transport. Depression of uptake of cations by decreased pH has been noted before with Ni^{2+} and Mn^{2+} in *Sacch. cerevisiae* (Rothstein & Hayes, 1956; Fuhrmann & Rothstein, 1968) and with Cu^{2+} in *A. pullulans* (Gadd & Griffiths, 1980a), and Babich & Stotzky (1977) found that the toxicity of cadmium to fungi was potentiated at pH 8 or 9.

The theory that intracellular accumulation of cadmium was a controlled event was supported by the stoichiometric exchange of Cd^{2+} and K^+ ions across the cell membrane for yeast-like cells. Potassium release has been associated with membrane disruption and loss of viability (Passow & Rothstein, 1960; Gadd & Mowll, 1983), though this was not necessarily true for *Sporobolomyces roseus* (Mowll & Gadd, 1983), but in this study the evidence suggested that potassium efflux occurred to maintain the ionic balance across the cell membrane. Analysis of Lineweaver-Burk plots showed that cadmium uptake and potassium release did not occur at the same site and, in addition, ionophore-induced potassium release from yeast-like cells did not increase the rate of cadmium influx. Thus, the implication was that the antiporter model postulated for $\text{Ca}^{2+}/\text{K}^+$ exchange during Ca^{2+} efflux from *Sacch. cerevisiae* (Eilam, 1982) did not apply to the $\text{Cd}^{2+}/\text{K}^+$ exchange found in yeast-like cells of *A. pullulans*.

The efflux of intracellular cadmium, enhanced by NTA or glucose, was an indication that cadmium existed within the cell primarily in an ionic state, or bound to low molecular weight compounds, a fate suggested for zinc ions accumulated by *Candida utilis* (Failla *et al.*, 1976). A possible fate of cadmium ions within the cell was that postulated for cations within cells of *Saccharomyces carlsbergensis* where cations were accumulated in vacuoles (Okorokov *et al.*, 1977; Lichko *et al.*, 1982). Further work is needed to clarify this point.

This study has defined the nature of cadmium uptake by *A. pullulans* and has also illustrated that yeast-like cells, mycelium and chlamydo-spores can tolerate levels of cadmium usually thought to be toxic to fungi and yeasts (Babich & Stotzky, 1977; Gadd & Mowll, 1983). The mechanism of tolerance involved exclusion of cadmium in chlamydo-spores and a restricted intracellular uptake of cadmium by yeast-like cells and mycelium. Though mycelium showed a slightly greater sensitivity, all cell types were tolerant to cadmium levels beyond reported phylloplane concentrations (Bewley & Campbell, 1978, 1980), and it is possible that yeast-like cells and mycelium are able to maintain viability and reproduce on metal-polluted phylloplanes, forming chlamydo-spores only when other stringent conditions, such as desiccation, dictate.

G. M. G. gratefully acknowledges the receipt of a grant from the Natural Environment Research Council (NERC GR/3/4520).

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