Biol Metals (1991) 4:100-106

BIOLOGY OF METALS

Silver tolerance and accumulation in yeasts

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Received September 12, 1990

Summary. *Debaryomyces hansenii* (NCYC 459 and strain 75-21), *Candida albicans* (3153A), *Saccharomyces cerevisiae* (X2180-1B), *Rhodotorula rubra* (NCYC 797) and *Aureobasidium pullulans* (IMI 45533 and ATCC 42371) were grown on solid medium supplemented with varying concentrations of $AgNO_3$. Although Ag^+ is highly toxic towards yeasts, growth on solid media was still possible at Ag concentrations of 1–2 mM. Further subculture on higher Ag concentrations (up to 5 mM) resulted in elevated tolerance. The extent of Ag tolerance depended on whether Ag-containing plates were exposed to light prior to inoculation since light-mediated reduction of Ag^+ to Ag^0 resulted in the production of a less toxic silver species. Experimental organisms exhibited blackening of colonies and the surrounding agar during growth on $AgNO_3$-containing medium especially at the highest Ag concentrations tested. All organisms accumulated Ag from the medium; electron microscopy revealed that silver was deposited as electron-dense granules in and around cell walls and in the external medium. X-ray microprobe analysis indicated that these granules were metallic Ag^0 although AgCl was also present in some organisms. Volatile and non-volatile reducing compounds were produced by several test organisms which presumably effected Ag^+ reduction to Ag^0.

Key words: Silver tolerance – Silver accumulation – Yeasts

Introduction

Despite the known toxicity of silver to microorganisms (Trevors et al. 1985; Trevors 1987; Ghandour et al. 1988), many organisms are resistant or tolerant to Ag despite considerable Ag accumulation by the cells (Charley and Bull 1979; Goddard and Bull 1989a, b). Some silver-resistant bacteria, fungi and yeasts have been isolated from polluted habitats, e.g. photographic effluents and mining wastes (Belly and Kydd 1982; Pumpel and Schinner 1986). However, the means by which microorganisms can survive and grow in the presence of potentially toxic concentrations of Ag has not been fully established, though environmental parameters play important roles. For example, Ag resistance in *Escherichia coli* and *Pseudomonas stutzeri* was dependent on the chloride concentration (Silver 1983; Gadd et al. 1989); high levels of external Cl^- reduced Ag^+ toxicity and levels of Ag accumulation because of the formation of insoluble AgCl (Gadd et al. 1989). Other binding interactions in the external environment or growth media may also contribute to a lower availability of Ag^+ (Tilton and Rosenberg 1978). Some Ag-resistant strains of bacteria accumulate low amounts of Ag (Haefeli et al. 1984; Kaur and Vadhera 1986; Starodub and Trevors 1989) though this can again be influenced by medium composition. Whatever the mechanism involved, survival in the presence of silver must depend on exclusion from the cells unless an internal detoxification mechanism is possessed, e.g. synthesis of metal-binding proteins or intracellular localization. In the yeast *Cryptococcus albidus,* silver markedly affected ultrastructural characteristics and deposition was observed in vacuoles (Brown and Smith 1976). In other organisms examined, predominantly bacteria, most of the accumulated silver was associated with cell walls, envelopes or other outer surfaces (Belly and Kydd 1982; Goddard and Bull 1989b).

There is little information relating to silver and yeasts, though a resistant *Rhodotorula* sp., as well as several black-pigmented fungi, were isolated from photographic sludge but not characterized further (Belly and Kydd 1982). The objective of this work was therefore to examine silver tolerance and accumulation in yeasts and determine the cellular location and form of the metal accumulated. A possible role for reduction of Ag^+ to elemental Ag^0 is also discussed.

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Materials and methods

Organisms, media and cultural conditions. The yeasts *Saccharomyces cerevisiae* X2180-1B, *Candida albicans* 3153A, *Rhodotorula rubra* NCYC 797, *Debaryomyces hansenii* NCYC 459 and strain 75-21 and the yeast-like fungus *Aureobasidium pullulans* IMI 45533 and ATCC 42371 were maintained on MYGP agar of composition ($g\ l^{-1}$): D-glucose, 10; yeast extract (Lab M), 3; malt extract (Lab M), 3; neutralized bacteriological peptone (Oxoid), 5; agar (Lab M no. 2), 15. Silver-containing plates were prepared by adding appropriate amounts from a 100 mM $AgNO_3$ stock solution to molten MYGP agar at 50–55° C. Poured plates were immediately covered and allowed to set in darkness. All incubations, unless otherwise stated, were carried out in the dark at 25° C.

Silver uptake. After growth on a range of $AgNO_3$ concentrations, yeast colonies were suspended in 5 ml of 5 mM 1,4-piperazinediethanesulfonic acid (Pipes) buffer, adjusted to pH 6.5 using solid tetramethylammonium hydroxide and centrifuged (1200 *g*, 10 min). Resulting cell pellets were washed three times with distilled water and finally resuspended in 8 ml distilled water. For dry mass determination, three 1-ml aliquots were removed and placed in pre-weighed aluminium foil cups and dried for 48 h at 105° C. After centrifuging the remaining 5 ml suspension (1200 *g*, 10 min), the pellet was digested with 0.5 ml 6 M HNO_3 at 100° C for 1 h. After cooling, 2.5 ml of distilled water was added and, after mixing, the suspension was centrifuged (1200 *g*, 10 min) to remove debris. Silver concentrations of cell digests or supernatants were determined using a Pye Unicam SP9 atomic absorption spectrophotometer with reference to appropriate $AgNO_3$ standards.

Electron microscopy and X-ray microanalysis. Control and silver-containing plates were inoculated with each organism and incubated, as described above, for 10 and 20 days, respectively. Purified agar (Oxoid) at 50° C was poured carefully over the yeast colonies and, after setting, small cubes were cut containing embedded yeast colonies. Cells were fixed in 2% (vol./vol. water) triple-distilled glutaraldehyde in 5 mM Pipes pH 6.5 for 2 h at room temperature, and then washed four times with 5 mM Pipes pH 6.5 (15 min/wash). Dehydration was through a 25–100% (by vol.) ascending series of ethanol in distilled water, samples being left overnight at each stage. Three transfers were made in absolute ethanol. Blocks were then transferred to a 50% (by vol.) mixture of L. R. Whyte resin in absolute ethanol and infiltrated for 2 days on a rotary mixer at room temperature. After infiltration for a further 2 days in 100% L. R. Whyte resin, the agar cubes were placed in gelatin capsules with fresh resin and polymerized at 60° C for 2 days. Sections were cut using a Reichert OMU-3 ultra-microtome, mounted on formvar-coated copper grids and examined using a Jeol-1200 EX transmission electron microscope in conjunction with a Link series II X-ray microanalysis system with LZ-5 detector. For removal and examination of silver granules produced in the agar medium, portions of agar (approximately 0.5 cm^3) from the dark zones around colonies of $AgNO_3$-grown organisms were transferred into 10 ml distilled deionized water and heated at 100° C until the agar had dissolved. After centrifugation (1200 *g*, 5 min), the black pellet was washed three times with distilled deionized water at 100° C and then dried overnight in an Edwards Pirani II freeze drier. After mounting on formvar-coated copper electron microscope grids, granules were examined as described above. Fresh preparations of Ag_2S and AgCl were obtained by mixture of equimolar solutions of $AgNO_3$ and either Na_2S or NaCl, respectively. Precipitates were washed thoroughly in distilled deionized water and examined as above.

Detection of volatile and non-volatile reducing compounds. MYGP plates containing 0.001% (mass/vol.) methylene blue and 0.005% (mass/vol.) resazurin were streaked with the required test organism, incubated and checked daily for colour changes. To test for volatile reducing compounds, test organisms were streaked onto MYGP agar with or without 1 mM $AgNO_3$. A 0.005% (mass/vol.) resaszurin plate was positioned over each inoculated plate and the whole system sealed with tape. The resazurin plate was observed weekly for colour change during incubation at 25° C.

Results

Silver accumulation

When *R. rubra* and *D. hansenii* were grown on $AgNO_3$-containing agar there was little difference in the amounts of Ag accumulated at different Ag concentrations (Table 1). *S. cerevisiae* X2180-1B accumulated significantly more Ag than the other yeasts, though again, there was little difference between values obtained at different Ag concentrations (Table 1). The two strains of *D. hansenii* were less Ag-tolerant than *S. cerevisiae* and *R. rubra*. Repeated subculture of strains on Ag-containing media ('training') resulted in increased levels of Ag accumulation by *R. rubra* whereas trained *S. cerevisiae* showed reduced Ag uptake. After 'detraining', by repeated subculture of trained strains on Ag-free media, amounts of Ag accumulated by *S. cerevisiae* increased and were similar to those recorded in control cells whereas values in detrained *R. rubra* showed little difference from trained cells and were still markedly higher than controls (Table 1). Ag tolerance was retained by *D. hansenii* NCYC 459 after the 'detraining' procedure (Table 1). The maximum amount of Ag taken up was approximately 90 μmol (g dry mass)$^{-1}$ in *R. rubra;* this corresponds to approximately 1% of the dry mass of cells.

For three of the four yeasts examined, there was no significant difference in Ag uptake whether grown on light-exposed plates or in the dark (Table 2). With *R. rubra* however, there was a marked increase in Ag uptake on light-exposed plates (Table 2). Furthermore, with all the organisms tested (the yeast strains shown in Tables 1 and 2 as well as *C. albicans* 3153A, *A. pullulans* IMI 45533 and ATCC 42371) there was an increase in biomass production, i.e. reduced Ag toxicity; it can be seen in Table 2 that both strains of *D. hansenii* and *S. cerevisiae* X2180-1B could grow at 4 mM and 5 mM $AgNO_3$ in light-exposed plates whereas 3 mM $AgNO_3$ was the maximum concentration tolerated in dark-incubated plates (Table 2). Light-exposed plates were of much darker appearance than those manipulated in the dark because of photoreduction of Ag^+ to Ag^0 (Greenwood and Earnshaw 1984).

Electron microscopy and X-ray microanalysis

All organisms exhibited blackening of colonies when grown on $AgNO_3$-containing medium, particularly around edges and on the underside; dark zones were also evident in the surrounding agar (Fig. 1). Electron microscopic examination of Ag-grown organisms revealed the presence of electron-dense granules within or around cell walls and in the external medium (Figs.

Table 1. Silver accumulation by yeasts grown on solid MYGP agar containing $AgNO_3$

Yeast	$AgNO_3$ concn. in medium (mM)	Ag accumulated by cells [μmol Ag (g dry mass)$^{-1}$]		
		control strains	trained strains	detrained strains
Rhodotorula rubra (NCYC 797)	0.5	2.3± 1.1	1.5± 0.5	1.8± 0.8
	1.0	4.9± 0.1	4.4± 0.8	6.3± 3.3
	2.0	9.4± 1.5	29.5± 6.4	19.2±14.3
	3.0	11.0	81.4±13.9	16.3± 5.3
	4.0	8.3± 3.6	92.1±34.3	56.9±20.9
	5.0	13.5	12.7± 6.2	17.7± 3.3
Debaryomyces hansenii (NCYC 459)	0.5	4.2± 1.1	3.1± 0.5	3.0± 0.5
	1.0	5.9± 0.1	8.2± 2.2	10.8
	2.0	ng	11.6± 4.1	nd
	3.0	ng	nd	15.8
	4.0	ng	16.3± 2.4	16.3
Debaryomyces hansenii (strain 75-21)	0.5	2.7± 0.4	2.6± 0.4	3.5± 0.7
	1.0	5.0± 0.4	5.9± 1.1	5.4± 0.5
	2.0	ng	10.5± 0.4	ng
	3.0	ng	9.2± 2.6	ng
Saccharomyces cerevisiae (X2180-1B)	0.5	29.8±14.5	14.6± 3.2	23.2± 0.6
	1.0	30.7±12.3	10.1± 1.8	31.0±11.0
	2.0	23.4± 7.2	19.7± 1.1	19.5± 0.1
	3.0	30.6	25.1± 3.9	ng

Trained strains were sub-cultured on Ag-containing medium at least ten times prior to Ag analysis; detrained strains were sub-cultured on Ag-free medium at least five times prior to re-inoculation onto Ag-containing medium and subsequent Ag analysis. All cells were harvested after a 3-week incubation at 25° C in the dark. Where possible, values are shown ±SEM. ng = no growth; nd = not determined

2, 3). Little or no granular deposits were observed within cells. X-ray microprobe analysis confirmed the presence of silver in granules associated with the cells or those recovered from dark zones in the agar around $AgNO_3$-grown colonies (Fig. 4). Comparison with authentic AgCl and Ag_2S indicated that the granules were elemental Ag^0 although some AgCl was associated with *D. hansenii* (75-21) while Ag_2S was possibly present in *C. albicans* 3153A (Fig. 4b, c). Microprobe analysis of a granule preparation taken from agar in the vicinity of colonies of *A. pullulans* IMI 45533 again indicated elemental Ag^0. It should be noted that a peak for Au (derived from the electron microscope) may partly overlap with the S peak (Fig. 4b).

Detection of reducing compounds

Methylene blue was reduced from blue to light green in the close vicinity of the colonies and this was most apparent and occurred mostly rapidly (within a week) with *D. hansenii* (NCYC 459 and strain 75-21), *R. rubra* (NCYC 797) and both strains of *A. pullulans* (IMI 45533 and ACTC 42731). The resazurin plates onto which the test organisms were streaked directly changed colour from purple/blue to fluorescent pink within 2 days. This colour change was least apparent with *S. cerevisiae* X2180-1B. After 1 month, the top resazurin plates in the detection system for volatile reducing compounds had also changed to a fluorescent pink with all organisms except *S. cerevisiae* X2180-1B. A positive result was achieved with all six yeasts, even when the base plate was silver-free, which suggested that the organisms normally produced these compounds in the absence of Ag.

Discussion

Silver is very toxic towards microorganisms yet growth of the test organisms was still possible at millimolar $AgNO_3$ concentrations in agar medium. Undoubtedly a considerable proportion of free Ag^+ was bound by medium constituents (Gadd and Griffiths 1978; Trevors 1987). However, organisms were still able to accumulate Ag, maximal values approaching 90 μmol (g dry mass)$^{-1}$ for *R. rubra*. This value corresponds to 9.6 mg (g dry mass)$^{-1}$ ($\approx$1% of the dry mass) which is higher than some previously reported values for yeasts, the mean value for six strains being 0.46 mg (g dry mass)$^{-1}$ (Pumpel and Schinner 1986). However, in the latter study, Ag accumulation was assessed after only 3 h incubation in culture medium containing 0.15 mM $AgNO_3$ (Pumpel and Schinner 1986). Certain bacteria appear to accumulate more Ag than yeasts and values of up to 28 mmol (g dry mass)$^{-1}$ [=3000 mg (g dry mass)$^{-1}$] have been reported for a multispecies community (Charley and Bull 1989). In non-growing cultures of *Citrobacter intermedius* B6, maximum Ag uptake of 4.35% (by mass) resulted at an initial Ag concentration

Table 2. Effect of light exposure on silver accumulation by trained strains of yeast grown on solid MYGP agar containing $AgNO_3$

Yeast	$AgNO_3$ concentration in medium (mM)	Ag accumulated by cells [μmol (g dry mass)$^{-1}$]	
		light-exposed plates	dark incubation
Rhodotorula rubra (NCYC 797)	0.5	1.2± 0.2	2.2± 0.1
	1.0	5.6± 3.3	6.1± 0.5
	2.0	28.8± 1.3	7.2± 0.5
	3.0	30.8± 2.0	7.3± 0.5
	4.0	62.7± 8.6	13.3± 6.2
	5.0	47.7	nd
	6.0	7.8	ng
Debaryomyces hansenii (NCYC 459)	0.5	2.8± 0.5	3.8± 1.1
	1.0	9.6± 0.5	10.1
	2.0	13.6	13.0± 2.7
	3.0	17.5	9.0
	4.0	10.9	ng
	5.0	8.3	ng
Debaryomyces hansenii (strain 75-21)	0.5	0.6±0.1	3.9± 0.9
	1.0	1.0±0.1	7.0± 2.2
	2.0	15.5±1.4	11.2
	3.0	8.3±1.4	11.8
	4.0	19.8±1.7	ng
	5.0	5.7	ng
Saccharomyces cerevisiae (X2180-1B)	0.5	15.4± 1.7	18.4± 0.6
	1.0	19.5± 4.4	22.3± 6.6
	2.0	27.3± 0.4	32.1
	3.0	25.9±11.7	29.8±14.7
	4.0	47.2	ng
	5.0	59.1	ng

One set of plates was exposed to light (12 μE m^{-2} s^{-1}) for 24 h prior to inoculation and incubation at 25° C for 3 weeks before being analysed for Ag; where possible, values are shown ±SEM. ng, no growth; nd, not determined. Other plates were incubated in the dark

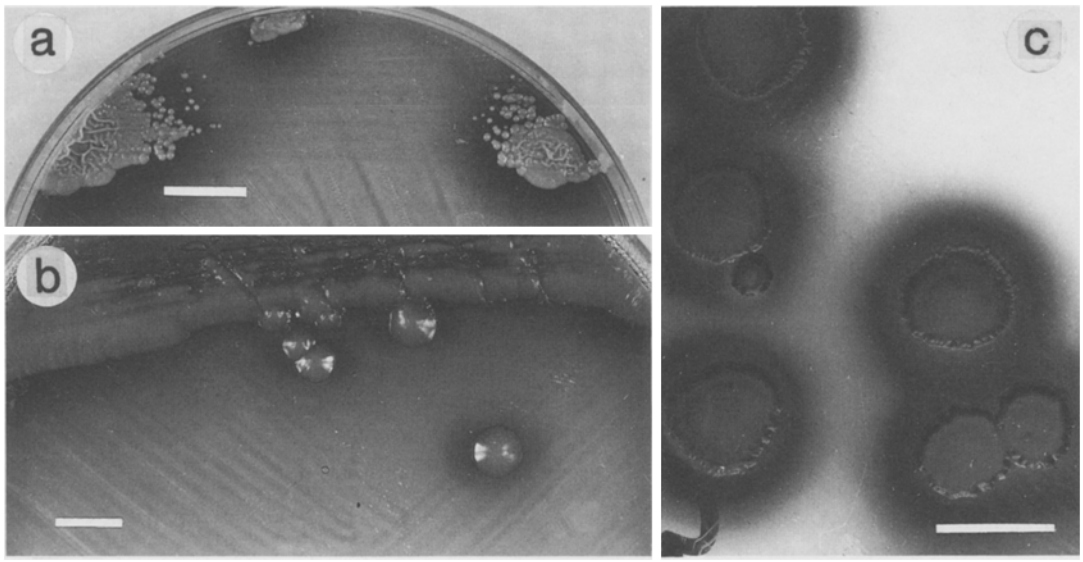

Fig. 1. Photographs of (**a**) *C. albicans* 3153A, (**b**) *R. rubra* NCYC 797 and (**c**) *A. pullulans* IMI 45533 growing on $AgNO_3$-containing agar medium. The $AgNO_3$ concentration was 3 mM and incubation was for approximately 3 weeks at 25°C in the dark. Bar markers indicate 1 cm

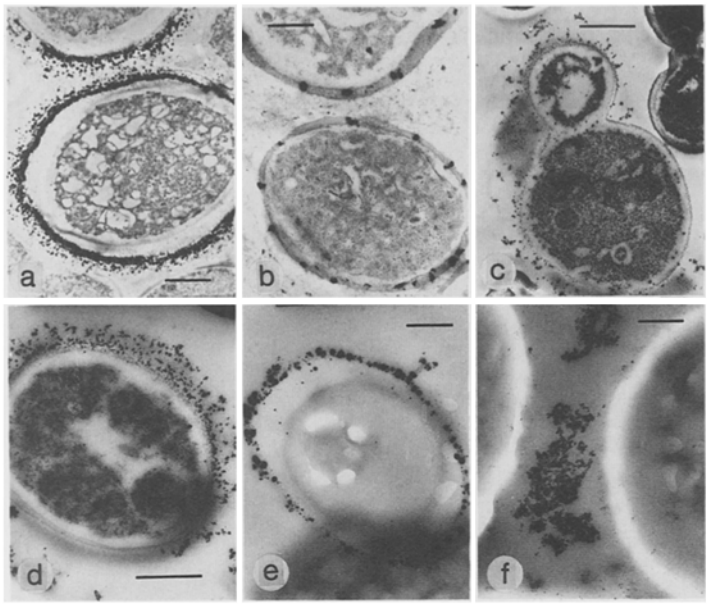

Fig. 2. Transmission electron micrographs of silver accumulation by yeast strains grown on Ag-containing agar (**a–f**). Note electron-dense granules/precipitation within and around cell walls. (**a, b**) *A. pullulans* IMI 45533 (**c, d**) *D. hansenii* (75-21) (**e**) *C. albicans* 3153A (**f**) *R. rubra* NCYC 797 showing silver precipitation between cells, possibly in extracellular polysaccharide. Cultures were grown for approximately 3 weeks at 25° C in the dark; $AgNO_3$ concentration in the agar was 4 mM for *A. pullulans* and *C. albicans*, 5 mM for *D. hansenii* and *R. rubra*. Most sections were stained using uranyl acetate/lead citrate according to standard electron microscopy protocols; (**e**) and (**f**) were unstained to emphasise the electron-dense silver deposits. Bar markers indicate (**a, b, c**) 1 μm, (**d**) 500 nm, (**e, f**) 200 nm

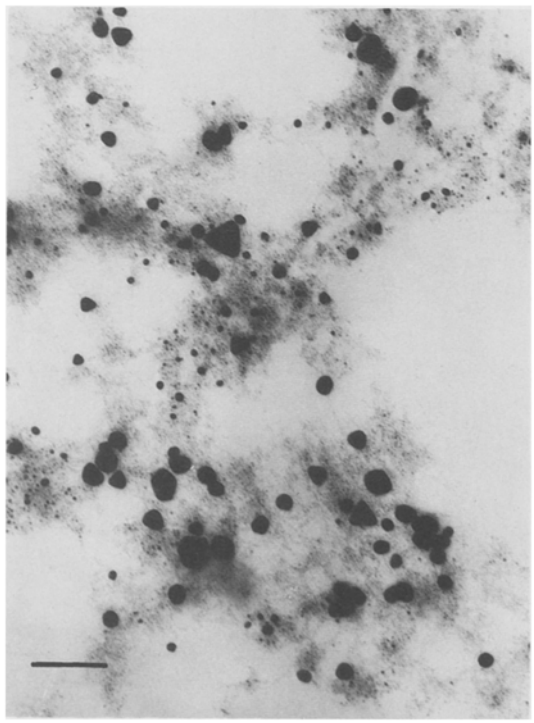

Fig. 3. Transmission electron micrograph of granules taken from agar in the vicinity of colonies of *A. pullulans* IMI 45533 grown for 3 weeks in the dark at 25° C on MYGP agar containing 3 mM $AgNO_3$. Bar marker indicates 200 nm

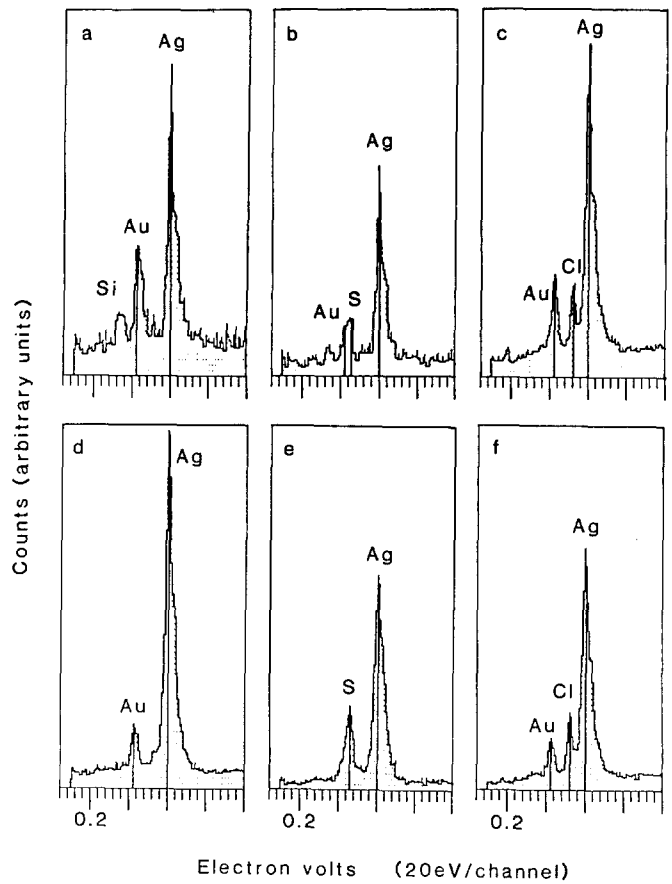

Fig. 4. X-ray microprobe analysis of electron-dense granules around yeast cells after growth in the presence of $AgNO_3$. (**a**) *D. hansenii* (75-21); (**b**) *C. albicans* 3135A; (**c**) large granule around *D. hansenii* (75-21); (**d**) granules extracted from agar medium in the vicinity of colonies of *A. pullulans* (IMI 45533); (**e**) Ag_2S; (**f**) AgCl. The peaks for Ag, S and/or Cl are indicated on the spectra. Note the contaminating Au peak which may partly overlap with the S peak

of approximately 2 mM; maximum uptake in growing cultures was 2.81% (by mass) at an effective Ag concentration of approximately 0.2 mM (Goddard and Bull 1989b). For a series of bacterial strains tolerant to >0.1 mM Ag^+, a mean Ag accumulation value of 213 μmol (g dry mass)$^{-1}$ was recorded (Pumpel and

Schinner 1986), while a mean Ag accumulation value of 224 μmol (g dry mass)$^{-1}$ was recorded for 32 strains of filamentous fungi (Pighi et al. 1989). However, meaningful comparisons are difficult since, in uptake studies, there may be no uniform standardisation of suspension density; hence, the significance of any extracellularly produced polysaccharide as a biosorbent for Ag, or the effect of suspension medium components, is often unclear. For example, the presence of Cl^- can markedly affect Ag^+ concentration and bioaccumulation because of AgCl precipitation (Gadd et al. 1989). Furthermore, electron microscopy revealed that not all cells in a given population examined had accumulated significant amounts of Ag, as indicated by the formation of electron-dense granules in and around cell walls. Average values of Ag accumulation by the population do not take this cell heterogeneity into account. A similar phenomenon was observed for uranium accumulation by *S. cerevisiae* where the average value of U biosorption was approximately 15% of the dry mass. However, not all cells accumulated U (which was deposited as layers of needle-like fibrils around the cells) and the proportion of U in accumulating cells approached 50% of the dry mass (Strandberg et al. 1981). Goddard and Bull (1989a, b) also observed an uneven distribution of electron-dense silver deposits in bacterial populations. It was suggested that this was either due to population heterogeneity in respect of Ag accumulation and/or attrition during growth in the bioreactors. The latter hypothesis can be discounted in our study since the yeast colonies were fixed and embedded *in situ* and few or no granules were removed from the cells during preparatory procedures.

There was little evidence for intracellular accumulation of Ag and the majority of cell-associated Ag was present as electron-dense granules within and around cell walls, or sometimes associated with extracellular polysaccharide as in *R. rubra*. There was no apparent vacuolar Ag deposition as reported for *Cryptococcus albidus* (Brown and Smith 1976). Most of the Ag accumulated by cells could be removed by washing with dilute acids, which again indicated little internalization of the metal. X-ray microprobe analysis confirmed the presence of Ag in the granules and comparison with spectra obtained for Ag_2S and AgCl indicated that the granules were predominantly elemental Ag^0, although some AgCl and Ag_2S were detected in some samples. However, the chemical reactivity of Ag^+ is such that AgCl and Ag_2S are always likely to be present when Ag^+ is introduced into undefined growth media. Other indirect evidence for production of elemental Ag^0, probably by reduction of Ag^+, was obtained using light-exposed plates. Photoreduction of Ag^+ to Ag^0 (Greenwood and Earnshaw 1984) resulted in a darkening of plates due to the formation of Ag^0 granules; such granules were also observed in the dark haloes around yeast colonies growing on $AgNO_3$-containing agar. Purified granules gave similar X-ray spectra to cell-associated granules. Other experiments revealed that most of the yeasts were capable of producing volatile and non-volatile reducing substances and these could effect transformation of Ag^+ to Ag^0. As previously mentioned, dark zones were evident around Ag-grown colonies and microscopic examination clearly revealed extensive silver granule formation in the agar. The production of reducing substances by a *Pseudomonas* isolate has been reported previously and, during growth in the presence of silver, metallic silver was deposited in colonies and on glass surfaces in culture flasks (Belly and Kydd 1982). Such a response results in detoxification of Ag^+ because Ag^0 is a less toxic form; this process clearly occurs in the yeast strains examined here. Such a detoxification was also confirmed by the higher concentrations of Ag apparently tolerated in plates where prior photoreduction of Ag^+ to Ag^0 had taken place before inoculation. It should be noted that reducing substances were produced by strains growing in the absence and presence of Ag. Detoxification of Ag^+ by reduction to elemental Ag^0 is therefore not an induced phenomenon but rather a 'gratuitous mechanism of resistance' (Gadd and Griffiths 1978). However, some degree of 'training' of yeast strains to increasing Ag concentrations in agar was possible, perhaps by selection of cells with enhanced capacities for Ag^+ reduction.

Acknowledgement. G. M. G. gratefully acknowledges support from the University of Dundee Research Initiatives Fund. M. K. gratefully acknowledges receipt of a European Social Fund training grant for biotechnology.

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