Journal of Industrial Microbiology (1995) 14, 300–311

Reduction of selenium oxyanions by unicellular, polymorphic and filamentous fungi: cellular location of reduced selenium and implications for tolerance

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(Received 30 March 1994; accepted 1 October 1994)

Key words: Fungi; Yeasts; Selenium; Selenite; Selenate; Reduction; Tolerance

SUMMARY

The ability of several filamentous, polymorphic and unicellular fungi to reduce selenite to elemental selenium on solid medium was examined. *Fusarium* sp. and *Trichoderma reesii* were the only filamentous fungi, of those tested, which reduced selenite to elemental selenium on Czapek–Dox agar resulting in a red colouration of colonies. Other organisms (*Aspergillus niger*, *Coriolus versicolor*, *Mucor* SK, and *Rhizopus arrhizus*) were able to reduce selenite only on malt extract agar. Several fungi were able to grow in the presence of sodium selenite but were apparently unable to reduce selenite to elemental selenium, indicating that other mechanisms of selenite tolerance were employed, such as reduced uptake and/or biomethylation to less toxic, volatile derivatives. Sodium selenate was more toxic to *Fusarium* sp. than selenite, and the toxicity of both oxyanions was increased in sulphur-free medium, with this effect being more marked for selenate. Scanning electron microscopy of *Aspergillus funiculosus* and *Fusarium* sp. incubated with sodium selenite showed the presence of needle-like crystals of elemental selenium on the surfaces of hyphae and conidia, while transmission electron microscopy of *A. funiculosus* revealed the deposition of electron-dense granules in vacuoles of selenite-treated fungi. Several yeasts were able to grow on MYGP agar containing sodium selenate or sodium selenite at millimolar concentrations. Some, notably *Rhodotorula rubra* and *Candida lipolytica*, and the polymorphic fungus *Aureobasidium pullulans* were also effective at reducing selenite to elemental selenium, resulting in red-coloured colonies. *Schizosaccharomyces pombe* was able to grow at selenite concentrations up to 5 mmol L^{-1} without any evidence of reduction, again indicating the operation of other tolerance mechanisms.

INTRODUCTION

The metalloid selenium is in the same group in the Periodic table as sulphur and the two elements share some chemical properties. For example, the oxyanions selenate and selenite may be regarded as analogues of sulphate and sulphite, respectively. Selenium is now thought to be an essential biological element in animals, bacteria and plants [9,13]. Several proteins in animal and bacterial systems have been shown to contain functional selenocysteine (the selenium-containing analogue of cysteine), such as murine glutathione peroxidase and *Escherichia coli* formate dehydrogenase [2]. Selenocysteine has been proposed as the 21st proteinaceous amino acid [2]. However, the toxicity of inorganic selenium compounds, e.g. selenite and selenate, to plants, animals and microorganisms is also well known [5,14,24]. There is some evidence for common metabolic routes for sulphur and selenium compounds in microorganisms, and in addition to selenocysteine, selenium analogues of sulphur-containing amino acids, e.g. selenomethionine and selenocystine have been found in bacterial systems [22]. Uptake of selenate and selenite in plants is thought to occur via different processes [5,14]. Recent information on selenate and selenite uptake by microorganisms is sparse, though studies on *Salmonella typhimurium* [4] and *Selenomonas ruminantium* [11] indicated that selenate uptake was mediated by the sulphate transport system while selenite transport was distinct from the former process.

Fungi, as well as other microorganisms, transform inorganic selenium compounds to methylated derivatives, e.g. dimethyl selenide, which are volatile and less toxic than inorganic forms [9,16]. Biomethylation of selenium by fungi has received much attention and has been used for the bioremediation of selenium-contaminated soils and waters [25]. In addition to methylation, fungi such as *Aspergillus parasiticus* [17], *Fusarium* sp. [21] and *Mortierella* spp. [31] can reduce selenate and selenite to elemental selenium (Se^o), a process which also results in detoxification [10,15]. Early studies showed that intact cells of *Candida albicans* rapidly reduced selenite to Se^o, the latter being accumulated by cells. Reduction was inhibited by metabolic poisons such as formate and dinitrophenol [8]. In cell-free extracts of *C. albicans* and *Saccharomyces cerevisiae* enzymatic reduction of selenite could be restored in dialyzed preparations by the addition of glucose-6-phosphate, triphosphopyridine nucleotide and oxidized glutathione [18]. It was suggested that selenite was bound to sulphydryl groups of proteins and was released as Se^o upon accepting four electrons. Little recent information on fungal selenite reduction is available though several bacteria have been shown to conserve energy via the reduction of selenate;

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these organisms may reduce selenate, either to selenite or directly to elemental selenium; selenite can be further reduced to elemental selenium [15,30]. In addition, Oremland et al. [19,20,23] demonstrated dissimilatory selenate reduction to elemental selenium by anaerobic bacteria in sediments via $SO_4^{=}$ independent respiration.

In the present study, a range of filamentous fungi and yeasts were screened for the ability to reduce selenite to Se^0 in order to assess whether this was a widely occurring phenomenon in fungi, such findings being of relevance to an understanding of the significance of this process in the natural environment as well as the suitability of certain strains for any biotechnological applications [16]. The effect of sulphate in the growth medium on selenite toxicity and reduction by filamentous fungi was also evaluated. Organisms capable of high levels of selenite reduction were examined by scanning electron microscopy to determine the cellular site(s) of Se^o deposition. The implications of these findings for selenium tolerance are discussed.

MATERIALS AND METHODS

Organisms and culture conditions

Filamentous fungi (*Aspergillus funiculosus*, *Aspergillus niger*, *Coriolus versicolor* C7B 863A, *Fusarium* sp. *Mucor hiemalis* IMI 21216, *Mucor* SK, *Penicillium chrysogenum* IMI 178514, *Penicillium funiculosum* IMI 114933, *Rhizopus arrhizus* IMI 57412 and *Trichoderma reesii* GH532) were maintained on malt extract agar plates (50 g L^{-1}, LabM (Amersham), Bury, Lancs, UK). The polymorphic fungus *Aureobasidium pullulans* IMI 45533, and the yeasts *Candida albicans* 3153a and 75–59, *Candida glabrata* 733–36 and C33, *Candida lipolytica* 37–1, *Debaryomyces hansenii* NCYC 459 and 793, *Rhodotorula rubra* NCYC 797, *Saccharomyces cerevisiae* X2180-1B, 6175/11a and NCYC 744, *Schizosaccaromyces pombe* 972h$^-$ and *Zygosaccharomyces rouxii* NCYC 1522 were maintained on MYGP agar, consisting of (g L^{-1}) malt extract (LabM) (3.0), yeast extract (LabM) (3.0), bacteriological peptone (LabM) (5.0), D-glucose (BDH) (10.0) and agar (LabM No. 2) (15.0). All organisms were cultured in the dark at 25 °C.

Screening for selenite reduction and toxicity

Incubation of filamentous fungi with sodium selenite was carried out on Czapek–Dox agar, consisting of (g L^{-1}) sucrose (30.0), $NaNO_3$ (2.0), KH_2PO_4 (1.0), $MgSO_4{\cdot}7H_2O$ (0.5), KCl (0.5), and agar (LabM No. 2) (15.0), sulphur-free Czapek–Dox agar (with $MgCl_2{\cdot}6H_2O$ replacing magnesium sulphate) or malt extract agar (LabM). Sodium selenite (Na_2SeO_3) was added to media at 50–55 °C from a stock solution (1 mol L^{-1}) in distilled, deionized water. Filamentous fungi growing on malt extract agar plates were inoculated into sulphur-containing Czapek–Dox agar and incubated in the dark at 25 °C for 10 days. Disks (6-mm diameter) were cut from the margins of the resulting colony under aseptic conditions; these disks wre inoculated into the centre of duplicate test plates containing the appropriate medium plus sodium selenite (0–10 mmol L^{-1}). Test plates were incubated as above for 14 days. The ability of filamentous fungi to reduce selenite was measured visually, the degree of red colouration (due to the presence of amorphous elemental selenium) being used as an indication of reduction. It should be noted that the absence of red colour does not necessarily mean that selenite is not reduced since reduction to selenide or formation of complexes other than amorphous Se^o may be possible [14].

For growth experiments, 6-mm disks taken from the margins of a culture growing on Czapek–Dox agar (8-day-old) were inoculated into the centre of Czapek–Dox agar plates (prepared with or without sulphate) containing 0–50 mmol L^{-1} sodium selenite or 0–10 mmol L^{-1} sodium selenate. Plates were incubated as above for 14 days. Radial growth measurements were made daily during the incubation period. Yeasts growing on MYGP agar were streaked on MYGP agar plates containing 0–5 mmol L^{-1} sodium selenite. Plates were incubated in the dark at 25 °C for at least 8 days.

Electron microscopy and X-ray microprobe analysis

To prepare filamentous fungi for scanning electron microscopy, small cylinders (about 0.2 cm^3) of agar (± selenite)/fungal biomass were cut from plates using a sterile cork borer. These were fixed in 2% (v/v) glutaraldehyde (SEM grade) in 5 mmol L^{-1} 1,4-piperazine diethanesulphonic acid (PIPES) buffer, adjusted to pH 6.5 using solid tetramethylammonium hydroxide, for 2 h at room temperature. Fixed colonies were washed four times in 5 mmol L^{-1} PIPES (15 min per wash) prior to dehydration in an ethanol/distilled water series (25%, 50%, 75% and 100% (v/v)), samples being incubated overnight in each stage. Following dehydration, samples were taken through an acetone/ethanol series (20%, 40%, 60%, 80% and 100% acetone (v/v)); three transfers were made in absolute acetone. The cylinders (submerged in acetone) were critical point dried using a Polaron (Polaron (Fisons) Instruments, East Grinstead, W. Sussex, UK) E3000 series II critical point drying apparatus after exposure for one hour in the transitional fluid (liquid CO_2). Dried samples were mounted on aluminium stubs and sputter-coated with palladium-gold alloy using a Polaron E1500 coating unit. Samples were finally examined using a Jeol JSM 35C scanning electron microscope (Jeol (UK) Ltd, Welwyn Garden City, Herts, UK) with an accelerating voltage of 25 kV. Samples for X-ray microprobe analysis were fixed, dehydrated and dried as above, but were examined uncoated using a Cambridge 360 scanning electron microscope (Cambridge Instruments, Cambridge, UK) in conjunction with a Link Systems (Link Systems, High Wycombe, Bucks, UK) X-ray microprobe system.

Fungal colonies were prepared for transmission electron microscopy according to the method of Kierans et al. [12]. Agar (LabM No. 2) at 50–55 °C was carefully poured over fungal biomass on Czapek–Dox agar plates (± sodium selenite). After setting, cylinders (about 0.4 cm^3) were cut using a sterile cork borer. These were fixed in glutaraldehyde, washed and dehydrated in an ethanol: water series in the same way as samples for scanning electron microscopy. Three transfers were made in absolute ethanol prior to infiltration in 25%(v/v) LR White (Agar Scientific, Stansted, Essex, UK) resin in ethanol on a rotary mixer overnight at room tempera-

ture. This was followed by infiltration in 50%(v/v) LR White resin in ethanol and a final infiltration in 100% LR White resin under the same conditions. The cylinders were then placed in gelatine capsules with fresh LR White resin and polymerized at 60 °C overnight. Ultrathin sections were cut using a Reichert OMU-3 microtome (Leica (UK) Ltd, Milton Keynes, Bucks, UK), and mounted on formvar-coated copper grids prior to staining them with uranyl acetate and lead citrate. The sections were finally examined using a Jeol 1200 EX transmission electron microscope.

RESULTS

Toxicity of sodium selenite and reduction to elemental selenium by filamentous fungi

Selenite treatment inhibited growth (measured in terms of colony diameters attained after 14 days incubation) of most of the filamentous fungi tested on all media used (Table 1). Inhibition of fungal growth generally increased with increasing selenite concentration. Even at the lowest concentration of sodium selenite (1 mmol L^{-1}), most of the fungi incubated on sulphur-containing Czapek–Dox agar (CDA) showed a marked reduction in colony diameter compared with control plates. *Coriolus versicolor* and *Rhizopus arrhizus*, however, showed significant growth on this medium containing 1 mmol L^{-1} selenite, with colony diameters equal to or approaching control values after 14-day incubation. *Trichoderma reesii* was the only species tested that was capable of significant growth in the presence of 10 mmol L^{-1} selenite; the colony diameter attained after 14 days at this selenite concentration was, surprisingly, greater than that on lower concentrations. Inhibition of growth was not markedly greater on sulphur-free CDA than on the sulphur-containing medium for most of the filamentous fungi studied. However, it should be emphasized that these point measurements may not reflect changes in growth rate or possible lag phases and growth after the 14-day incubation period (see Figs 1 and 2). Only *Mucor* SK showed greater inhibition of growth on sulphur-free medium than on sulphur-containing medium when treated with 1 mmol L^{-1} sodium selenite, while the growth of *T. reesii* was more greatly affected by 10 mmol L^{-1} sodium selenite on sulphur-free medium. It is likely that fungi still exhibited growth on sulphur-free medium because of stored sulphur compounds, trace SO_4 and/or S-containing amino acids in the medium. There were variable effects of the use of malt extract agar (MEA) on growth inhibition by selenite. Growth of some organisms was more inhibited by selenite on MEA than on less complex media, particularly *Aspergillus niger*, *Mucor* SK and *M. hiemalis*. *T. reesii*, however, showed much greater tolerance to selenite on MEA than on more defined media, and the colony diameter of this organism on 1 and 5 mmol L^{-1} selenite after 14 days on MEA was the same as the controls.

Few of the filamentous fungi grown on CDA showed the ability to reduce selenite to elemental selenium (Se°), i.e. produce a red or orange colouration of colonies. However, colonies of *Fusarium* sp. and, to a lesser extent, *T. reesii*, grown on CDA showed a red colouration in the presence of selenite. While control cultures of *Fusarium* sp. produce a red colouration, the red colouration was markedly greater on selenite-containing media with some changes in colony morphology, notably a corralloid appearance of colony margins on 5 mmol L^{-1} sodium selenite. Colonies of *Penicillium chrysogenum* were pink when grown on selenite-containing CDA; no such colouration was observed in control plates. The absence of sulphur in the growth medium appeared to decrease the ability of these organisms to reduce selenite. When sulphur-free CDA was used as the test medium, the red colouration of *Fusarium* sp. was diminished when compared with sulphur-containing CDA at all selenite concentrations. At 10 mmol L^{-1} selenite, *T. reesii* colonies were not red on sulphur-free CDA. However, colonies of *M. hiemalis* showed more red colouration when grown on sulphur-free CDA containing 5 mmol L^{-1} selenite than on the corresponding sulphur-containing medium.

Several of the filamentous fungi reduced selenite when MEA was used as the test medium, despite considerable inhibition of radial growth at higher selenite concentrations. *M. hiemalis* colonies were an intense red colour in the presence of 1 and 5 mmol L^{-1} selenite on MEA, but little growth beyond the inoculation plug had occurred after 14 days incubation. Similar results were obtained with *Coriolus versicolor*, *Penicillium chrysogenum* and *P. funiculosum*. *Fusarium* sp. and *T. reesii* both reduced selenite to Se° on MEA; in the case of *T. reesii*, the degree of red colouration of colonies was greater on MEA than on CDA.

In all cases where evidence of reduction of selenite was observed, the red colouration was confined to the fungal colony, with little or no colouration of the medium.

The effects of selenate and selenite on growth of Fusarium *sp. on solid media*

Fusarium sp. reduced selenite to amorphous elemental selenium on sulphur-free and sulphur-containing CDA, and this organism was therefore chosen for more detailed growth studies with selenate and selenite. Sodium selenate was inhibitory to the growth of *Fusarium* sp. at concentrations less than 10 mmol L^{-1} on both sulphur-containing and sulphur-free CDA (Fig. 1(A,B)). On sulphur-containing CDA, selenate concentrations up to 1.0 mmol L^{-1} reduced the radial growth rate of this organism, but the colony diameters attained after 14 days were the same as in control experiments (Fig. 1(A)). At 2–5 mmol L^{-1} sodium selenate, the radial growth rate was more significantly affected, and the colony diameter attained after 14 days was about 30% of controls. The effect of selenate on growth was greater on sulphur-free CDA than on sulphur-containing CDA (Fig. 1(B)). At low concentrations (0.1 and 0.2 mmol L^{-1}), there was a period of growth for 3 days, followed by cessation of growth. Growth resumed after 7 days at growth rates only slightly lower than those measured in control experiments. At higher concentrations of sodium selenate, growth ceased after 3 days incubation and no further increase in colony diameter was noted.

Sodium selenite was much less toxic than sodium selenate to *Fusarium* sp. Radial growth of *Fusarium* sp. on sulphur-containing CDA was only slightly affected by 1 or 5 mmol L^{-1} sodium selenite (Fig. 2(A)), though moderate inhibition of

TABLE 1

Colony growth and reduction of sodium selenite by filamentous fungi on solid media after 14 days incubation at 25 °C

Organism	Selenite concentration (mmol L^{-1})	CDA		Sulphur-free CDA		MEA	
		Reduction	Colony diameter (mm)	Reduction	Colony diameter (mm)	Reduction	Colony diameter (mm)
Aspergillus niger	0		85.0 ± 0.0		85.0 ± 0.0		85.0 ± 0.0
	1	–	37.3 ± 3.2	–	36.1 ± 1.5	+	14.1 ± 0.6
	5	–	18.2 ± 1.2	–	16.6 ± 0.3	–	9.8 ± 0.6
	10	–	16.0 ± 1.3	–	10.0 ± 0.7	–	6.0 ± 0.0
Coriolus versicolor C7B 863A	0		85.0 ± 0.0		85.0 ± 0.0		85.0 ± 0.0
	1	–	66.1 ± 1.2	–	66.7 ± 1.1	+	85.0 ± 0.0
	5	–	6.0 ± 0.0	–	41.3 ± 1.8	+	6.0 ± 0.2
	10	nd	nd	nd	nd	+	5.9 ± 0.2
Fusarium sp.	0		85.0 ± 0.0		85.0 ± 0.0		85.0 ± 0.0
	1	+++	27.7 ± 3.1	++	42.0 ± 1.1	++	39.2 ± 0.3
	5	+++	20.3 ± 0.4	++	25.4 ± 0.6	++	23.4 ± 1.2
	10	+++	8.7 ± 1.0	++	8.6 ± 0.5	–	6.2 ± 0.3
Mucor hiemalis IMI 21216	0		85.0 ± 0.0		85.0 ± 0.0		85.0 ± 0.0
	1	–	38.4 ± 1.7	+	59.0 ± 2.4	–	10.8 ± 2.5
	5	+	7.6 ± 0.5	++	8.2 ± 1.1	+++	10.8 ± 0.5
	10	nd	nd	nd	nd	+++	10.6 ± 0.5
Mucor SK	0		85.0 ± 0.0		85.0 ± 0.0		85.0 ± 0.0
	1	–	85.0 ± 0.0	–	35.6 ± 0.8	–	6.7 ± 0.3
	5	–	9.6 ± 1.1	–	6.3 ± 0.3	+++	6.1 ± 0.1
	10	–	7.2 ± 0.5	–	5.9 ± 0.0	+++	6.4 ± 0.2
Penicillium chrysogenum IMI 178514	0		85.0 ± 0.0		64.9 ± 1.6		85.0 ± 0.0
	1	+	21.7 ± 2.3	–	37.6 ± 2.0	++	14.1 ± 1.0
	5	+	15.1 ± 0.9	+	14.8 ± 1.7	++	7.9 ± 0.3
	10	+	7.6 ± 0.3	–	8.1 ± 0.6	++	8.5 ± 0.4
Penicillium funiculosum IMI 114933	0		55.4 ± 7.2		53.3 ± 7.2		85.0 ± 0.0
	1	+	17.3 ± 1.3	–	25.4 ± 2.2	+	12.2 ± 0.5
	5	–	8.6 ± 0.2	–	11.4 ± 0.5	+	11.2 ± 0.9
	10	nd	nd	nd	nd	+	8.4 ± 0.8
Rhizopus arrhizus IMI 57412	0		85.0 ± 0.0		85.0 ± 0.0		85.0 ± 0.0
	1	–	85.0 ± 0.0	–	85.0 ± 0.0	++	85.0 ± 0.0
	5	–	6.1 ± 0.2	–	7.9 ± 1.0	–	7.8 ± 0.8
	10	–	7.7 ± 0.4	–	6.7 ± 0.3	–	8.0 ± 1.0
Trichoderma reesei GH532	0		85.0 ± 0.0		85.0 ± 0.0		85.0 ± 0.0
	1	++	26.2 ± 2.4	+	21.1 ± 2.1	++	85.0 ± 0.0
	5	++	21.3 ± 1.0	–	17.8 ± 1.6	++	85.0 ± 0.0
	10	++	39.8 ± 2.9	–	8.0 ± 0.8		38.4 ± 2.5

CDA, Czapek–Dox agar; MEA, malt extract agar. Fungi growing on sulphur-containing CDA plates were inoculated on to the appropriate agar medium (containing the appropriate concentration of selenite) as 6-mm disks from the margins of the culture. After 14 days incubation at 25 °C, the colony diameter was measured (four measurements) and the fungi assessed for selenite reduction (degree of red colouration, +++ (red), ++ (orange) or + (pink). nd = not determined; – (no colouration).

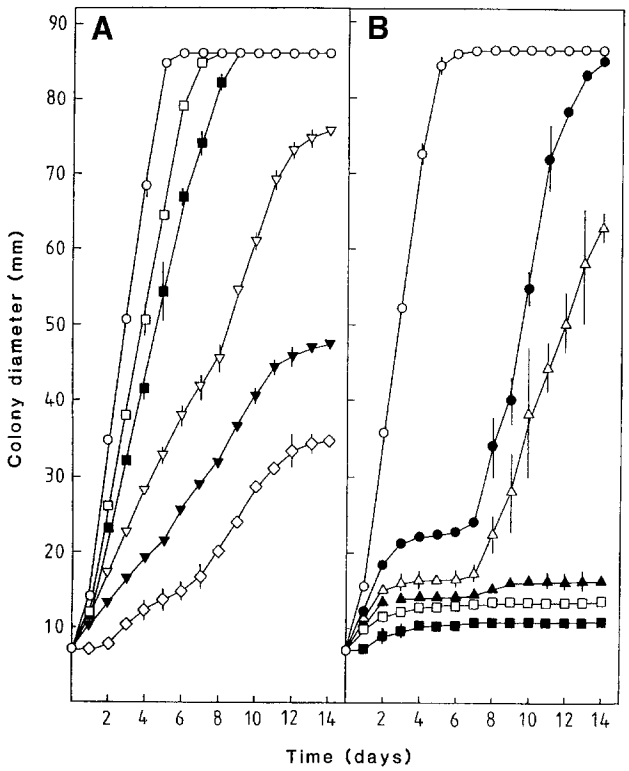

Fig. 1. Growth of *Fusarium* sp. on (A) sulphur-containing and (B) sulphur-free Czapek–Dox agar containing 0.0 (○); 0.1 (●); 0.2 (Δ); 0.4 (▲); 0.6 (□); 1.0 (■); 2.0 (▽); 3.0 (▼); or 5.0 (◇) mmol L^{-1} sodium selenate. Disks (6-mm diameter) taken from the margins of *Fusarium* sp. cultures growing on sulphur-containing Czapek–Dox agar were inoculated on plates containing the appropriate medium/selenate concentration and incubated at 25 °C. Colony diameters were measured daily during the incubation period. Bars indicate standard error of the mean (six measurements) and when not shown were smaller than the symbol dimensions.

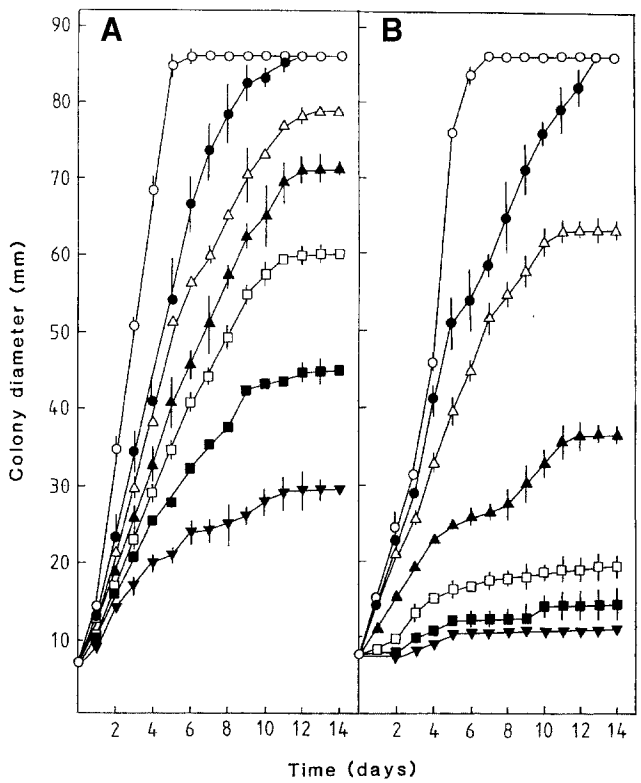

Fig. 2. Growth of *Fusarium* sp. on (A) sulphur-containing and (B) sulphur-free Czapek–Dox agar containing 0.0 (○); 1.0 (●); 5.0 (Δ); 10.0 (▲); 25.0 (□); 50.0 (■); and 100.0 (▼) mmol L^{-1} sodium selenite. Experimental conditions as in Fig. 1.

growth was measured on sulphur-free CDA at these concentrations (Fig. 2(B)). On sulphur-free CDA containing 5 mmol L^{-1} selenite, maximum growth of *Fusarium* sp. was measured after 10 days; the colony diameter was 71% of the control value after this time. On sulphur-containing CDA, the colony diameter of *Fusarium* sp. grown on 5 mmol L^{-1} selenite was 82% of the control value after 10 days and growth continued to the edge of the plate. At higher concentrations of selenite, the radial growth rate and colony diameter after 14 days were progressively affected on sulphur-containing CDA (Fig. 2(A)). On sulphur-free CDA, the effect of higher concentrations of selenite on radial growth rate and colony diameter at 14 days was, again, more marked than on sulphur-containing CDA (Fig. 2(B)) though the effect was not as great as with sodium selenate (Fig. 1(A,B)). At 50 mmol L^{-1}, growth was almost totally inhibited on sulphur-free CDA, though some growth was measured at this concentration on sulphur-containing CDA, albeit at a significantly reduced rate (Fig. 1(A)).

Toxicity of selenite and reduction to elemental selenium by yeasts and the polymorphic fungus Aureobasidium pullulans

A range of growth responses of yeasts to sodium selenite was observed on MYGP agar (Table 2). Growth of several yeasts: *Candida albicans* 3153a, *Saccharomyces cerevisiae* X2180-1B and 6175/11a, *Zygosaccharomyces rouxii* NCYC 1522 on 5 mmol L^{-1} selenite approached, or equalled, that of control cultures. Certain yeasts were able to grow moderately well on lower concentrations on selenite but were more affected at 5 mmol L^{-1} selenite, such as *Candida glabrata* C33 and *Candida lipolytica* 37-1, while inhibition of growth of some yeasts was measured at 0.5 and 1.0 mmol L^{-1} selenite, e.g. *C. glabrata* 733–36, *C. albicans* 75–59, *Rhodotorula rubra* NCYC 797 and *Aureobasidium pullulans* IMI 45533. Almost all of the organisms tested on MYGP agar reduced selenite to Se^{o}. Several *Candida* sp. and *Saccharomyces* sp. colonies had a pink colouration on all concentrations of selenite, while *C. lipolytica* 37–1 and *R. rubra* NCYC 797 were very effective reducers, producing bright red colonies on all concentrations of selenite. Colonies of *R. rubra* NCYC 797 grown in the absence of sodium selenite were red/pink; incubation on sodium selenite-containing medium produced a significantly darker red colouration. *A. pullulans* colonies were light pink on 1 mmol L^{-1} selenite and red on 5 mmol L^{-1} selenite. Only *Schizosaccharomyces pombe* 972h^{-} showed no colouration of colonies on selenite-containing medium. As with the filamentous fungi, the red colouration was confined to the yeast colonies, with little or no colouration of the medium being observed.

TABLE 2

Growth and reduction of sodium selenite by yeasts and *Aureobasidium pullulans* IMI 45533 on MYGP agar after 8-day incubation at 25 °C

Organism	Selenite concentration (mmol L^{-1})	Reduction	Growth
Aureobasidium pullulans IMI 45533	0		++++
	1	++	+++
	5	+++	++
Candida albicans	0		++++
3153a	0.5	+	++++
	1	+	++++
	5	+	++++
C. albicans	0		++++
75–59	0.5	+	+
	1	++	++
	5	++	++
C. glabrata	0		++++
C33	0.5	+	+++
	1	+	+++
	5	+	+++
C. glabrata	0		++++
733–36	0.5	+	+++
	1		
	5	+	+
C. lipolytica	0		++++
37–1	0.5	+++	++++
	1	+++	++++
	5	+++	++
Debaryomyces	0		++++
hansenii	0.5	–	–
NCYC 793	1	–	–
	5		
Rhodotorula	0		++++
rubra	0.5	+++	+++
NCYC 797	1	+++	++
	5	+++	+
Saccharomyces	0		++++
cerevisiae	0.5	+	++++
X2180-1B	1	+	++++
	5	+	++++
S. cerevisiae	0		++++
6175/11a	0.5	+	++++
	1	+	++++
	5	+	++++
S. cerevisiae	0		++++
NCYC 744	0.5	+	+++
	1	+	+++
	5	+	+++

Continued

TABLE 2 continued

Organism	Selenite concentration (mmol L^{-1})	Reduction	Growth
Schizosaccharomyces	0		++++
pombe	0.5	–	++++
972 h^-	1	–	++++
	5	–	–
Zygosaccharomyces	0		++++
rouxii	0.5	+	++++
NCYC 1522	1	+	++++
	5	+	++++

Yeasts were inoculated from cultures growing on MYGP agar plates onto MYGP agar containing the appropriate concentration of sodium selenite. After incubation at 25 °C for 8 days, the plates were assessed for growth (control = ++++; – = no growth) and reduction of selenite (degree of red colouration, +++ (red), ++ (orange), + (pink), – (no colouration)).

Incubation of selected yeast strains on MYGP agar containing sodium selenate or selenite showed that several yeasts also tolerated high concentrations of both oxyanions in this medium (Table 3).

Electron microscopic studies of Fusarium *sp. and* Aspergillus funiculosus

Examination of *Fusarium* sp. incubated on CDA containing 50 mmol L^{-1} selenite by scanning electron microscopy (SEM) revealed the presence of needle-like crystals on the surface of mycelia (Fig. 3(A,B)). Crystals were also observed on the surfaces of conidia (Fig. 3(C)), though these crystals were thicker and appeared to be more condensed than those deposited on the surfaces of mycelia. No such crystals were observed in selenite-free controls (Fig. 3(D)).

Incubation of *Aspergillus funiculosus*, another filamentous fungus capable of selenite reduction, on CDA containing 50 mmol L^{-1} selenite also resulted in the deposition of crystals on hyphae (Fig. 3(E)), though these were somewhat different to the crystals observed with *Fusarium* sp. (Fig. 3(A,B)). The

TABLE 3

Growth of yeasts and *Aureobasidium pullulans* on MYGP agar containing sodium selenite or selenate

Oxyanion concentration (mmol L^{-1})	*R. rubra* NCYC 797	*C. albicans* 3153a	*S. cerevisiae* X2180-1B	*D. hansenii* NCYC 459	*A. pullulans* IMI 45533
Selenite					
0.5	+++	++++	+++	+	+++
1.0	+++	+++	+++	+	+++
5.0	++	+++	+++	–	++
10.0	+	++	++	–	++
25.0	+	++	++	–	+
50.0	+	+	+	–	–
100.0	–	+	–	–	–
Selenate					
0.5	++++	++++	+++	++++	++++
1.0	+++	+++	+++	+++	+++
5.0	++	+++	++	+++	++
10.0	++	++	++	+++	++
25.0	++	++	++	++	++
50.0	++	+	+	+	+

Yeasts and *A. pullulans* were streaked on MYGP agar containing sodium selenite or selenate at the appropriate concentration and incubated at 25 °C for 10 days. Growth was compared with that attained on MYGP agar containing no selenium oxyanion (= ++++); – = no growth.

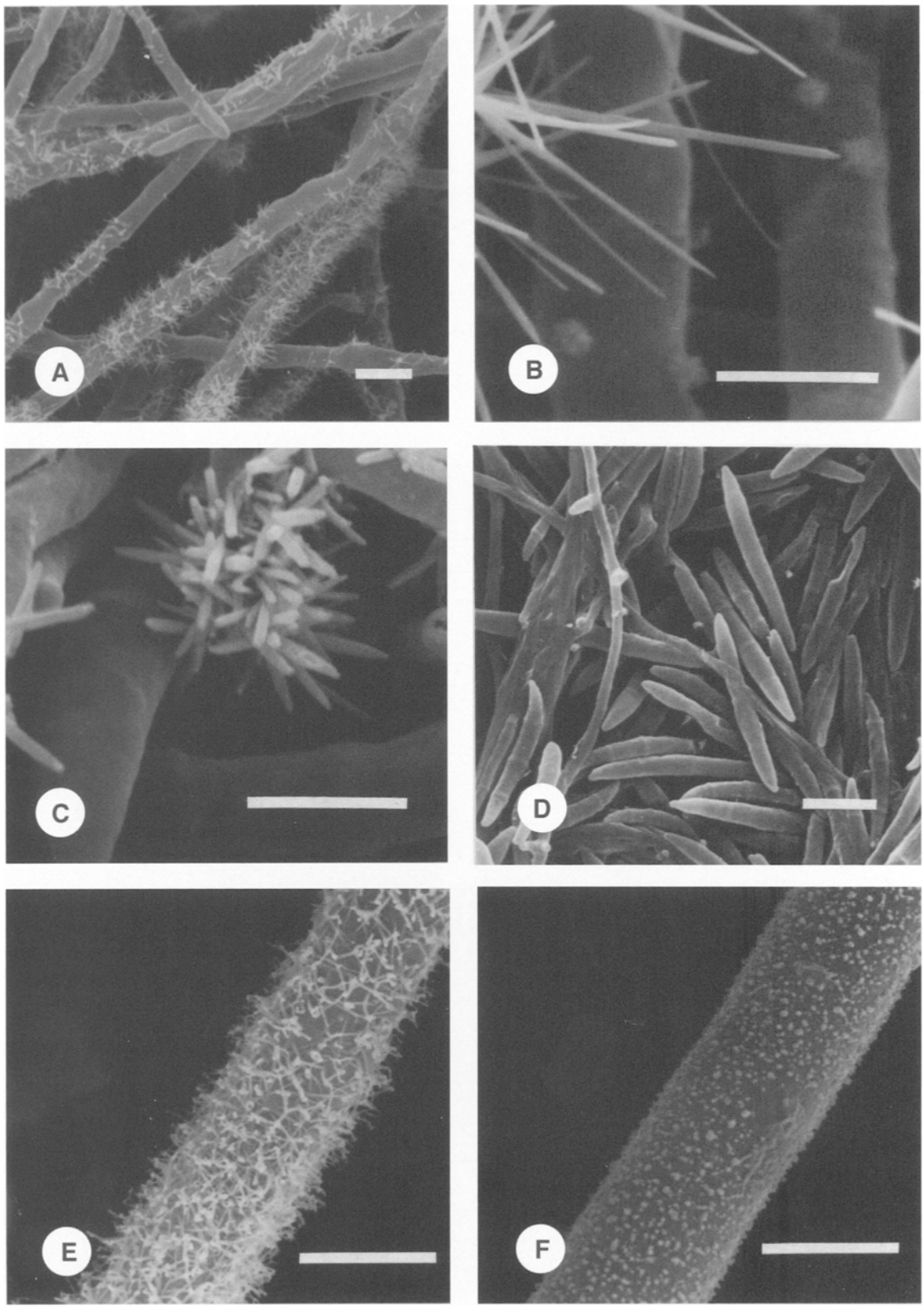

Fig. 3. Scanning electron micrographs of fungi: (A,B) hyphae and (C) conidia of *Fusarium* sp. incubated on Czapek–Dox agar containing 50 mmol L^{-1} sodium selenite for 10 days at 25 °C; (D) *Fusarium* sp. grown in the absence of selenite; (E) hypha of *Aspergillus funiculosus* incubated on Czapek–Dox agar containing 50 mmol L^{-1} sodium selenite for 10 days at 25 °C and (F) *A. funiculosus* grown in the absence of selenite. Bar markers (A,D,E,F) = 10 μm, (B,C) = 5 μm.

crystals on *A. funiculosus* were more thread-like and intertwined, in contrast to the needle-like structures observed on the surfaces of *Fusarium* sp. Again, these crystals were absent in *A. funiculosus* grown in the absence of selenite (Fig. 3(F)). X-ray microprobe analysis (XRMA) of the crystals observed in both organisms confirmed them to contain elemental selenium (Fig. 4(A,B)).

Transmission electron microscopy (TEM) of *A. funiculosus* ultrathin sections revealed the presence of electron dense granules located within vacuoles of fungi grown on CDA containing 50 mmol L^{-1} selenite (Fig. 5(A,B), as well as confirming the presence of thread-like crystals on the surfaces of hyphae. Neither structure was observed in sections of this fungus grown on selenite-free medium (Fig. 5(C)).

DISCUSSION

The toxicity of selenite to bacteria and algae is well known [6,13,27]. In this study, selenite was clearly inhibitory to the growth of the filamentous, polymorphic and unicellular fungi at millimolar concentrations, although some were able to grow at selenite concentrations up to 5 mmol L^{-1}. It should be made clear, however, that growth measurements were not taken after 14 days incubation. Therefore, it is possible that fungi incubated in the presence of selenite which had shown no growth after this time may have exhibited growth after further incubation. Only a few filamentous fungi showed the ability to reduce selenite to selenite to Se^{o} on CDA though more fungi were able to do this on MEA. The composition of the growth medium therefore affected reduction of selenite and, to a lesser extent, growth inhibition by selenite in filamentous fungi. Organisms which reduced selenite to Se^{o} on sulphur-contain-

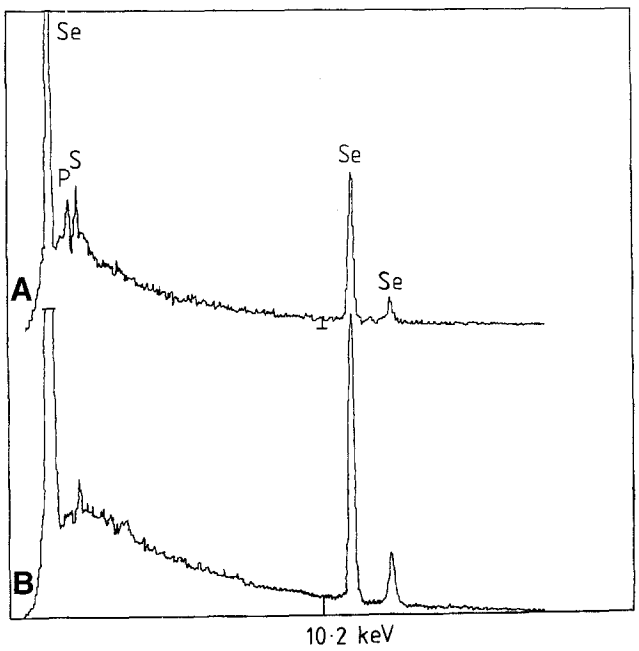

Fig. 4. X-ray microprobe analyses of crystals around (A) *Aspergillus funiculosus*, and (B) *Fusarium* sp. hyphae from fungi grown on sulphur-containing Czapek–Dox agar containing 50 mmol L^{-1} sodium selenite for 10 days at 25 °C.

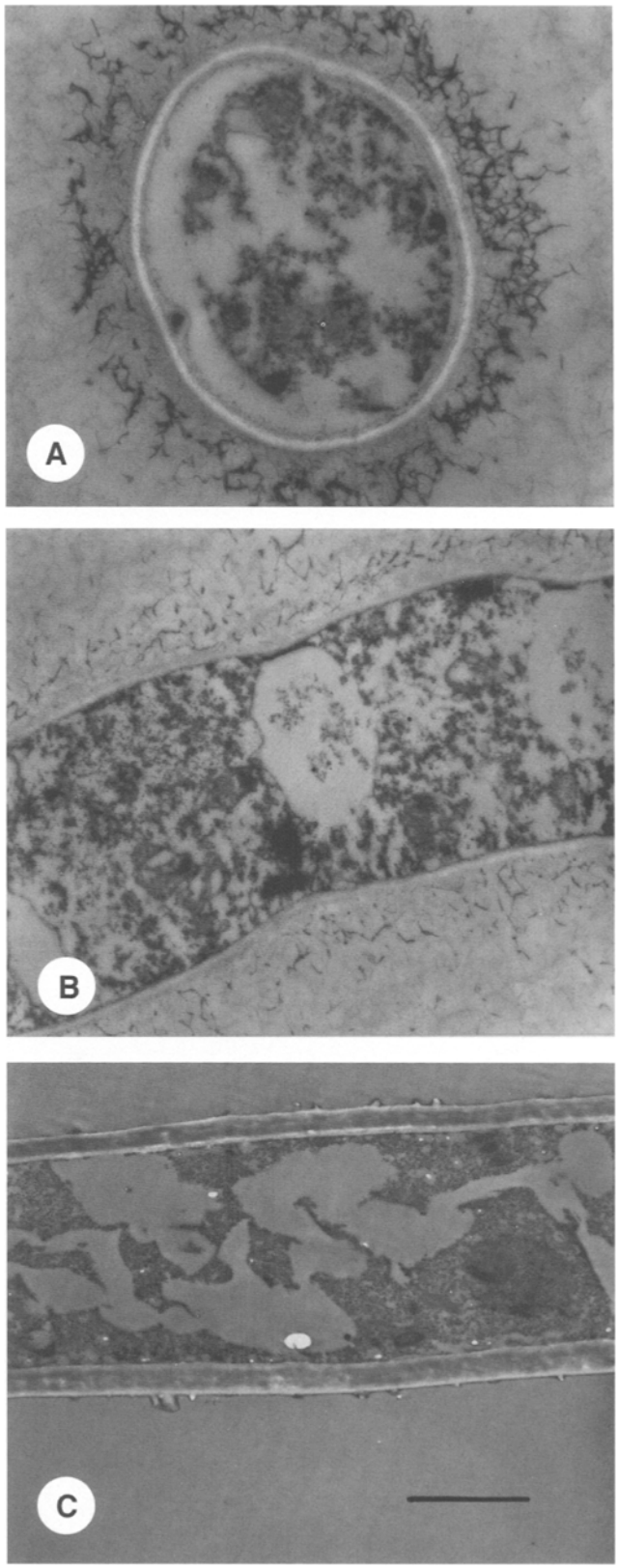

Fig. 5. Transmission electron micrographs of thin sections of hyphae of *Aspergillus funiculosus* grown on (A,B) sulphur-containing Czapek–Dox agar containing 50 mmol L^{-1} sodium selenite or (C) selenite-free sulphur-containing Czapek–Dox agar at 25 °C for 10 days. Bar marker = 2 μm.

ing CDA (*Fusarium* sp., *T. reesii*) showed a decreased ability to reduce selenite on sulphur-free CDA. However, the ability of *T. reesii* to reduce selenite appeared to be enhanced by incubation on MEA. This could be explained by the greater synthesis of selenite-reducing enzymes and/or metabolites on MEA. Reducing substances may be produced either in direct response to the presence of selenite or as a consequence of 'normal' metabolism. Nickerson and Falcone [18] demonstrated that triphosphopyridine nucleotide and reduced glutathione and glucose-6-phosphate were required for enzymic reduction of selenite in dialyzed extracts of *Candida albicans*. There have been few recent metabolic studies of selenite or selenate reduction in fungi, however. Bacteria conserve energy through the reduction of selenate [15,30]. Alternatively, there may be more complexation of selenite with the constituents of MEA than with those in CDA, thus attenuating the toxic effects of selenite in certain cases. The reduction of selenite to elemental selenium and accretion of the latter to cell surfaces has been regarded as a means of detoxification [21]. However, it is clear from the data in Table 1 that there was no direct relationship between the ability to reduce selenite and the ability to grow on selenite-containing media. For example, *Fusarium* sp. was clearly able to reduce selenite to Se°, but growth was markedly inhibited by selenite. Similarly, *Mucor hiemalis* colonies became an intense red colour when grown on MEA containing 10 mmol L^{-1} selenite, but little growth occurred beyond the inoculating plug. Conversely, *Coriolus versicolor* showed less inhibition of growth on CDA containing 1 mmol L^{-1} than most of the fungi studied, yet no evidence of selenite reduction was observed. It is likely that the tolerance of *C. versicolor* to selenite depended on a means of detoxification other than reduction, such as decreased uptake or methylation. Many of the fungi studied produced a garlic-like odour when incubated with selenite, indicating the presence of volatile derivatives of selenium, e.g. dimethyl selenide. Biomethylation of selenium as a means of detoxification is well documented [25], though it is unlikely that the high concentrations of selenite used in this study could all be removed by methylation. Therefore, while selenite reduction is an important mechanism of detoxification, it is not likely to be the only mechanism employed by the fungi examined in this study; consequently, the ability to grow on selenite does not necessarily reflect the ability to reduce selenite to Se°.

An unexpected finding was the apparent increase in toxicity of selenite in MEA when compared with CDA. Several organisms, notably *Aspergillus niger, Mucor* SK and *Mucor hiemalis*, showed greater inhibition of growth on the complete medium than on the more defined medium. The reasons for this are not clear. Two main mechanisms of selenite toxicity have been proposed, the affinity of selenite for sulphydryl groups in proteins, and the incorporation of selenium amino acid analogues, e.g. selenocysteine and selenomethionine, into proteins resulting in disruption of cellular biochemical activities [5,7]. It is possible that the uptake of selenite may be increased on MEA, and/or selenium incorporation into amino acids may be enhanced on complex media, resulting in an increase in toxicity by either or both mechanisms. In *Trichoderma reesii*, however, toxicity of selenite was much reduced on MEA when compared with growth on CDA.

Several of the yeasts were able to reduce selenite, as was the polymorphic fungus *Aureobasidium pullulans* IMI 45533 (Table 2). The *Candida* and *Saccharomyces* spp. were generally able to reduce selenite, producing pink colonies. Falcone and Nickerson [8] demonstrated the ability of *Candida albicans* RM 803 to reduce selenite to elemental selenium in liquid culture. Extracts of *Saccharomyces cerevisiae* also reduced selenite. Here, the most effective reducers of selenite were *Rhodotorula rubra* NCYC 797, *Candida lipolytica* 37–1 and *A. pullulans*, all of which produced bright red colonies on selenite-containing medium. A garlic-like odour was also produced by yeast cultures in the presence of selenite, indicating that these organisms are also capable of selenium methylation. One organism, *Schizosaccharomyces pombe* 972h$^-$, was able to grow at concentrations up to 5 mmol L^{-1} selenite without any evidence of reduction, indicating that other methods of selenite tolerance may be employed by yeasts as well as filamentous fungi.

The effect of selenite and selenate on the growth of *Fusarium* sp., one of the filamentous fungi able to reduce selenite, was studied in more detail. Selenate was much more toxic to this organism than selenite; the toxicity of selenate to microorganisms has been demonstrated [1,29]. The effect of sulphur in the growth medium was again important. Selenate toxicity was markedly increased when *Fusarium* sp. was grown on sulphur-free CDA; the effect of sulphur on the toxicity of selenite was less significant. Selenate/sulphate interaction in selenium toxicity is accepted [3,14] and it is believed that selenate uptake occurs via sulphate transport systems. Selenite transport is less well understood, though Tweedie and Segal [26] showed that selenite and selenate uptake was via the same mechanism in certain species of *Penicillium* and *Aspergillus*. Weissman and Trelease [28] demonstrated that the sulphur–selenium ratio was important in inhibition of mycelial growth of *A. niger* by selenate when sulphur was present as sulphate. Furthermore, selenate was converted to selenium analogues of amino acids in the absence of added sulphur, but remained in the inorganic state when methionine was present in the medium.

In the filamentous, polymorphic and unicellular fungi studied here, the red colouration apparently resulting from the deposition of elemental selenium was confined to the colony, indicating immobilization of selenium on cell material. Falcone and Nickerson [8] reported similar findings with *C. albicans* grown in liquid culture, though Ramadan et al. [21] observed colouration of a liquid culture of *Fusarium* sp., probably due to detached crystals of elemental selenium.

SEM and TEM revealed the presence of crystals of elemental selenium deposited on the surfaces of hyphae and conidia of *Fusarium* sp. and *Aspergillus funiculosus*, though the appearance of the crystals differed between these species. These findings complement those of Ramadan et al. [21] that elemental selenium is present on the surfaces of hyphae and conidia of fungi treated with selenite, though the hypothesis that this selenium is excreted from within the hyphae, as a means of reducing the intracellular concentration of selenium,

needs further confirmation. Ultrathin sections of *A. funiculosus* hyphae showed the presence of electron-dense granules in vacuoles of selenite-treated fungi which were absent in hyphae from untreated fungi suggesting that this organism has the ability to further compartmentalize elemental selenium. The role of the cell vacuole in the compartmentation of metal ions has long been regarded as important [10], though little or no information is available on metalloids.

In conclusion, this study has demonstrated the ability of several fungi to reduce selenite to elemental selenium and that deposition of selenium occurs both intra- and extracellularly. The composition of the growth medium used had a significant effect on selenite toxicity and reduction. However, reduction of selenium oxyanions to elemental selenium was not the only mechanism of tolerance employed by the fungi studied here. Mechanisms such as decreased uptake and volatilization due to biomethylation may also be important. In addition to being of environmental importance, fungal reduction of selenium oxyanions is also of biotechnological interest since it may result in the precipitation of elemental selenium from contaminated solution.

ACKNOWLEDGEMENTS

G.M. Gadd gratefully acknowledges receipt of a Scottish Office Education Department/Royal Society of Edinburgh Support Research Fellowship and financial support from the Biotechnology and Biological Sciences Research Council (GR/J48214). MMG gratefully acknowledges financial support from the Joint Egyptian/UK Government Channel Scheme. Grateful appreciation is also extended to Dr Pauline Handley (Department of Cell and Structural Biology, School of Biological Sciences, University of Manchester, UK) for carrying out the X-ray microprobe analysis and to Professor D. Lloyd (University of Wales College of Cardiff), Drs N.A.R. Gow (University of Aberdeen) and G.M. Walker (University of Abertay Dundee) for gifts of certain yeast strains. Thanks are also due to Martin Kierans and Ewan Starke (Department of Biological Sciences, University of Dundee) for technical assistance, including electron microscopy.

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