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Evidence for the involvement of vacuolar activity in metal(loid) tolerance: vacuolar-lacking and -defective mutants of *Saccharomyces cerevisiae* display higher sensitivity to chromate, tellurite and selenite

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The responses of *Saccharomyces cerevisiae* towards the oxyanions tellurite, selenite and chromate were investigated in order to establish the involvement of the yeast vacuole in their detoxification. Three mutants of *S. cerevisiae* with defective vacuolar morphology and function were used; mutant JSR180Δ1 is devoid of any vacuolar-like structure while ScVatB and ScVatC are deficient in specific protein subunits of the vacuolar (V)-H^+-ATPase. All the mutant strains showed increased sensitivity to tellurite and chromate compared to their parental strains. Such sensitivity of the mutants was associated with increased accumulation of tellurium and chromium. These results indicate that accumulation of both tellurium and chromium occurred mainly in the cytosolic compartment of the cell, with detoxification influenced by the presence of a functionally-active vacuole which may play a role in compartmentation as well as regulation of the cytostolic compartment for optimal expression of a detoxification mechanism, e.g. reduction. In contrast, the vacuolar-lacking mutant, JSR180Δ1, and the defective V-H^+ATPase mutant ScVatB displayed lower selenium accumulation than their parental strains. Additionally, the mutant strain ScVatB displayed a higher tolerance to selenite than the parental strain. This result suggests that accumulation of selenium occurs mainly in the vacuolar compartment of the cell with tolerance depending on the ability of the cytosolic component to reduce selenite to elemental selenium, which might, in turn, be related to activity of the V-H^+-ATPase. These results are discussed in relation to vacuolar compartmentation and the significance of the vacuolar H^+-ATPase in cytosolic homeostasis of H^+ both of which may affect the accumulation, reduction, and tolerance to the tested metal(loids).

Keywords: accumulation, metal(loids), toxicity, vacuole, vacuolar H^+-ATPase

Introduction

The tolerance of microorganisms to potentially toxic metals has received considerable attention in recent years. Although several microbial resistance and detoxification strategies include decreased uptake or impermeability (Gadd & White 1989), metal species can be intracellularly accumulated by fungi even from low external concentrations. Vacuoles are the largest intracellular compartment in yeasts and their functions include storage of amino acids (Matile *et al*. 1969), hydrolytic enzymes (Wiemken *et al*. 1979), and Ca^{2+} (Jones and Gadd 1990; Stevens 1992; Gadd 1995, Tanida *et al*. 1995). Additionally, a role for yeast vacuoles in the compartmentation and homeostasis of a wide range of toxic metal ions has been established (White & Gadd 1986; Klionsky *et al*. 1990; Gadd 1993) including Li^+ (Perkins & Gadd 1993a), Cs^+ (Perkins & Gadd 1993b), Mg^{2+} (Okorokov *et al*. 1980), Ni^{2+} (Joho *et al*. 1993), Fe^{2+} (Raguzzi *et al*. 1988; Bode *et al*. 1995) and Zn^{2+} (White & Gadd 1987). The vacuolar membrane has a specific H^+-translocating ATPase (V-ATPase) that

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generates an electrochemical gradient across the vacuolar membrane by pumping protons into the vacuole (Klionsky *et al*. 1990; Nelson *et al*. 1992). The electrochemical potential across the vacuolar membrane energizes the transport of cations and other substances into the vacuole (Gadd 1994). More recently, direct confirmation has been obtained for an essential role of the yeast vacuole in detoxification of Zn, Mn, Co and Ni (Ramsay & Gadd 1997). However, the involvement of the vacuole in compartmentation and detoxification of metal(loid) oxyanions has not been examined. The present investigation was carried out in order to examine the importance of functional yeast vacuoles in the tolerance and detoxification of the metal(loid) oxyanions, selenite, tellurite and chromate.

Materials and methods

Yeast strains

Saccharomyces cerevisiae mutant strains defective in vacuolar structure and function were used (Robinson *et al*. 1988). Three mutants were from a set isolated on the basis of deficient targeting of vacuolar proteins to the vacuole (vpt: vacuolar targeting mutant). Mutant JSR180Δ1 is devoid of vacuole-like structure(s) and was used as a model for vacuole-lacking yeast cells (Banta *et al*. 1988). This mutant strain was derived from the parental strain SEY 6210 (MATa, leu3-52, his3-D200, trp1-D901, lys2-801, suc2-D9, GAL) containing a carboxypeptidase Y-invertase-encoding plasmid (Banta *et al*. 1988). Mutants ScVatB and ScVatC are deficient in a specific protein subunit of the V-ATPase resulting in no acidification of the vacuole (Nelson & Nelson 1990). ScVatB lacks the B subunits of the catalytic sector and was derived from the parental strain W303-1B (MATa, leu2, his3, ade2, trp1, ura3) while ScVatC lacks the c proteolipid subunits of the membrane sector of the V-ATPase, and was derived from the parental strain W303-C (Nelson & Nelson 1990; Supek *et al*. 1994). Strains JSR180Δ1, SEY 6210, W303-C and ScVatC were kindly provided by Dr Scott Emr, University of California San Diego, USA, and strains W303-1B and ScVatB from Dr Lennart Adler, University of Goteborg, Sweden.

Media and culture conditions

All strains were routinely grown on MYGP agar of composition ($g\,l^{-1}$): malt extract (Lab M), 3.0; yeast extract (Difco), 3.0; bacteriological peptone (Oxoid), 5.0; D-glucose, 10.0 and agar (LabM, No.2), 15.0. The same composition without agar was used for liquid medium. Starter cultures were prepared by loop inoculating 50 ml of culture medium which was incubated for 24 h. These were then used to inoculate experimental cultures containing 50 ml medium to an initial OD_{550nm} of about 0.1. Strains were incubated for 24 h at 25 °C until the mid-exponential growth phase. For determination of the minimum inhibitory concentrations (MIC) and accumulated metal(loids), density gradient plates (Bryson & Szybalski 1952; Gadd *et al*. 1984; Horitsu *et al*. 1987) were prepared by pouring 60 ml of molten MYGP agar at 50 °C into 10 × 10 cm Petri dishes and allowing this to set at an angle of 30°. After solidifying, 60 ml of metal(loid)-amended agar was then poured onto the set base to give a linear metal gradient across the plate surface (Ramsay & Gadd 1997). Metal(loid) concentrations were prepared from sterile stock solutions of Na_2SeO_3, Na_2TeO_3 and K_2CrO_4 and appropriate volumes used to give final concentrations of 5.0 and 10.0 mM Na_2SeO_3, 2.5 mM Na_2TeO_3 and 1.0 mM K_2CrO_4. Plates were inoculated with 50 μl of each experimental culture which was streaked along the metal gradient using a sterile cotton wool swab. Plates were incubated at 25 °C for 5 days after which toxicity was monitored by the extent of growth along the gradient.

Measurement of accumulated metal(loids)

At the MIC of the more sensitive strain of each parental and mutant strain, cells were scraped from the surface of the Szybalski gradient plates after the incubation period and suspended in 5 mM PIPES buffer, pH 6.5. After washing the cells 3 × with the buffer, 5 ml of the buffer was added and cell numbers determined using a modified Fuchs-Rosenthal haemocytometer after appropriate dilution with ddH_2O. The cells were separated by centrifugation at 1200 *g* for 10 min. 1 ml of 6 M HNO_3 was added to the washed pellet and, after mixing, was incubated for 1 h at 90 °C after which 4 ml ddH_2O was added. Metal(loid) concentrations were measured using a Pye Unicam SP9 atomic absorption spectrophotometer (AAS) with reference to appropriate standard solutions.

Results

Toxicity of tellurite, chromate and selenite

Minimum inhibitory concentrations (MIC) of the studied metal(loids) were determined using a continuous linear concentration gradient (Bryson & Szybalski 1952; Ramsay & Gadd 1997) and are shown in Table 1. The MIC for tellurite for the mutant strains JSR180Δ1 and ScVatC were approximately half of those for the parental strains SEY6210 and W303-1B respectively. A similar result was also obtained for chromate although mutant strain ScVatB did not exhibit any significant difference in sensitivity to tellurite and chromate from its parental strain W303-1B. The mutant strain ScVatC also exhibited a higher sensitivity to selenite than its parent, W303-C. Mutant yeast strains JSR180Δ1 and ScVatB showed a different response towards

Table 1. Minimum inhibitory concentrations (MIC) expressed in μM chromate, tellurite or selenite, towards parental and vacuolar-deficient strains of *S. cerevisiae* determined using Szybalski density gradient plates. The concentration gradients in MYGP agar were 0.0–5.0 and 0–10.0 mM selenite, 0.0–2.5 mM tellurite and 0.0–1.0 mM chromate. Values shown are the mean of 5 replicates ± SEM

Yeast strain	Chromate	Tellurite	Selenite
SEY6210	999.0 ± 122.5	1292.0 ± 32.0	3302.0 ± 340.8
JSR180Δ1	466.2 ± 17.4	786.6 ± 86.1	3040.0 ± 334.4
W303-1B	842.4 ± 18.4	961.4 ± 61.7	3074.2 ± 478.3
ScVatB	716.4 ± 47.8	870.2 ± 49.8	5349.8 ± 299.9
W303C	644.4 ± 15.2	1155.0 ± 53.3	3877.9 ± 404.8
ScVatC	286.2 ± 31.0	706.8 ± 23.6	2580.2 ± 184.2

selenite. There were no great differences in MIC for the mutant JSR180Δ1 and its parental strain while strain ScVatB displayed tolerance to a higher selenite concentration than the parental strain W303-1B.

Accumulation of tellurium, chromium and selenium

The cellular content of tellurium, chromium and selenium was determined at the MIC of the most sensitive strain of each pair of *S. cerevisiae* strains (Figure 1). Accumulation of tellurium and chromium was increased significantly in the mutant strains. The vacuolar-lacking mutant JSR180Δ1 accumulated about five times more tellurium than its parental strain SEY6210 while mutant strain W303-C accumulated about four times more tellurium than the parental strain. Similarly, chromium accumulation was also significantly increased in the vacuolar-defective yeast strains ScVatB and ScVatC as well as in vacuole-deficient JSR180Δ1 compared to their corresponding parental strains (Figure 1a,b). Accumulation of selenium was different from tellurium and chromium and the vacuolar-lacking strains JSR180Δ1 and ScVatB accumulated less selenium than their parental strains SEY-6210 and W303-1B respectively. Additionally there was no appreciable difference in selenium accumulation between the mutant strain ScVatC and the matched strain W303-C (Figure 1c).

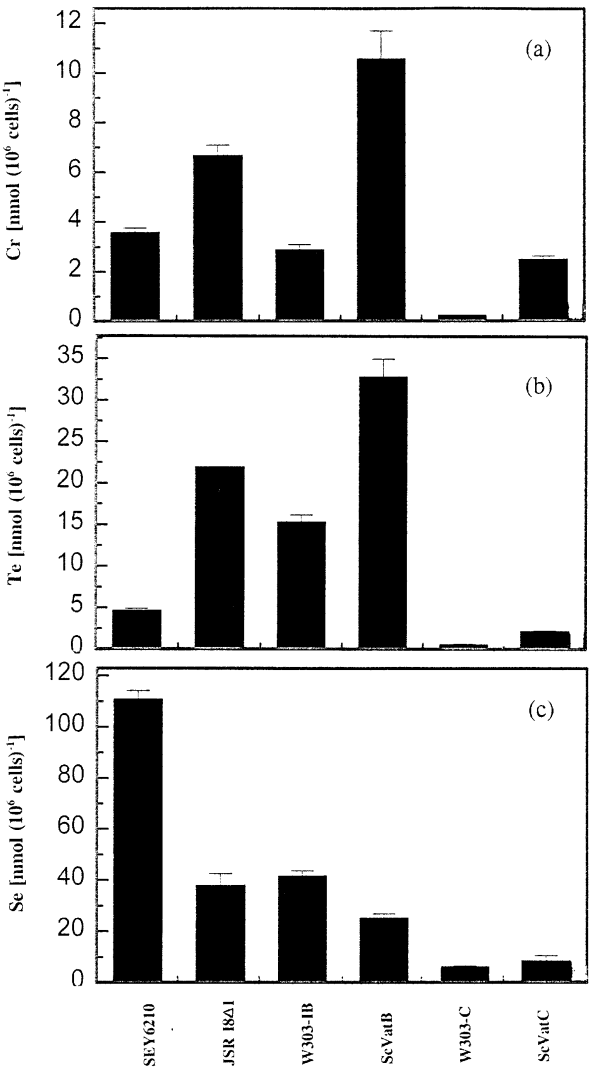

Figure 1. Accumulation of chromium, tellurium and selenium by the parental and vacuolar mutant strains of *S. cerevisiae*. Cells were harversted from locations on the plate corresponding to MIC values for the mutant strains (as described by Ramsay & Gadd 1997) after 5 days growth at 25 °C on metal-containing Szybalski density gradient plates. The metal content in acid-digested cell suspensions was determined by atomic absorption spectrophotometry. Each bar represents the mean of five replicates ± SEM.

Discussion

The relationship between metal uptake and toxicity has been documented in many instances since metal resistant microbial strains often exhibit an ability to prevent or reduce entry of toxic metal species into the cell (Gadd & White 1989). However, such a relationship is not universal as a Co^{2+}-resistant strain of *S. cerevisiae* had a higher cellular Co^{2+} content than the sensitive parental strain although this was because of compartmentation in the vacuole (White & Gadd 1986). In this investigation, both vacuolar-lacking and -defective mutants of *S. cerevisiae*

displayed higher sensitivity and accumulation of tellurium and chromium. This result suggests that the toxicity of both tellurite and chromate is manifest in the cytosolic compartment with the presence of completely structural and functional cellular vacuole(s) necessary to decrease the toxic action of these metalloids. Resistance of *Enterobacter cloacae* towards hexavalent chromium (chromate) was attributed to reduction to the trivalent form which is less toxic and which then precipitates (Ohtake *et al*. 1990). A chromate-sensitive mutant of *Pseudomonas ambigua* also reduced chromate(VI) much more slowly than the chromate-resistant wild type (Horitsu *et al*. 1987). Chromate reduction in a cell suspension of *Agrobacterium rodobacter* was related to the redox potential and it was found that chromate reduction required an energized cell membrane as the addition of depolarizing agents inhibited Cr(VI) reduction (Liovera *et al*. 1993). However a general mechanism of chromate resistance in the yeasts *Candida* sp. And *Rhodosporium* sp. was shown to be due to reduced uptake of chromate rather than reduction from Cr(VI) to Cr(III) (Pepi & Baldi 1993).

The mechanism of tellurite resistance in microorganisms is not completely understood. Several studies have reported transformation of tellurite to volatile methylated forms by bacteria and fungi (Konetzka 1977; Summers 1978; Karlson & Frankenberger 1993) and also the reduction to elemental tellurium which appears as black deposits inside or outside the cell (Corfield & Smith 1970; Taylor *et al*. 1988; Lloyd-Jones *et al*. 1994). In the present study, and despite the higher tellurium content of the mutant strains, growing colonies of the mutant strains appeared lighter in colour than the parental strains which were a much darker black colour. This may indicate a lower ability of the mutant strains to reduce tellurite to elemental tellurium which may be correlated with the higher sensitivity.

Selenium tolerance and detoxification by microorganisms has received prior attention and microbial transformations include (i) volatilization of inorganic selenium (more toxic) to volatile organic forms (less toxic) (Brady *et al*. 1996) and (ii) reduction of selenium oxyanions, selenite and selenate to inert elemental selenium (Se0) (Moss *et al*. 1987; Gharieb *et al*. 1994). The toxic action of selenium is believed to be due to incorporation into protein amino acids instead of sulfur which can lead to altered tertiary structure and dysfunction of proteins and enzymes (Lauchli 1993). Thus, avoidance of selenium incorporation into protein amino acids can be considered to be a detoxification mechanism. Our results show a coincidence of decreased toxicity with decreased cellular selenium. It is also worth mentioning that the red colour characteristic of reduction of selenite to elemental selenium appeared darker in the mutant strains JSR180Δ1 and ScVatB than their parental strains. The unexpected finding that the vacuolar-deficient strains were more tolerant to selenite may be due to the mutant strains being able to more efficiently transform intracellular and extracellular selenite to elemental selenium. Cytosolic enzymic reduction of selenium oxyanions is known and has been studied in both cell-free extracts and intact cells of *Candida albicans* and *S. cerevisiae* (Falcone & Nickerson 1963; Nickerson & Falcone 1963). Other recent studies have shown that a non-resistant culture of *Candida utilis* VSB-651 accumulated twice as much selenium than resistant *C. ethanolica* VSB-814 with the former organism having a higher activity of glutathione reductase and selenium-dependent glutathione peroxidase than *C. ethanolica* (Zhiltsova *et al*. 1996). Here, it was suggested that selenium accumulation by the yeasts was related to the activity of these oxido-reductive enzymes. In the present study, the vacuolar-lacking mutant JSR180Δ1 showed lower accumulated selenium, about 20% of found in the parental strain, while both strains displayed the same tolerance (MIC) to selenite. This observation suggests the involvement of the yeast vacuole in the regulation of both selenite uptake and detoxification. Since the V-ATPase regulates cytosolic pH through proton fluxes, this enzyme could affect indirectly the enzymatic reduction of selenite to elemental selenium in the cytosol. The absence of the vacuole or inactivation of the V-ATPase may gratuitously mean that the cytosolic pH is more suitable for activity of the redox enzymes that are responsible for selenite reduction to elemental selenium. In conclusion, this work has shown that accumulation of tellurium and chromium occurs mainly in the cytosolic compartment of the cell, with detoxification influenced by the presence of a functionally-active vacuole which may play a role in compartmentation as well as regulation of the cytosolic compartment for optimal expression of a detoxification mechanism, e.g. reduction. Accumulation of selenium occurs mainly in the vacuolar compartment of the cell with tolerance depending on the ability of the cytosolic component to reduce selenite to elemental selenium, which might, in turn, be related to activity of the V-H^+-ATPase. Tolerance to these metal(loids) therefore depends on cellular content and reduction in the cytosol which may be regulated by the presence of functionally active vacuoles.

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