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Modulation of chromium(VI) toxicity by organic and inorganic sulfur species in yeasts from industrial wastes

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Two chromium(VI) resistant yeast strains (*Candida* sp. and *Rhodosporidium* sp.) were isolated from industrial wastes. Four different yeasts, three from the Industrial Yeast Collection and one of pharmaceutical origin, were also studied in relation to chromate toxicity and its alleviation by sulfur species. The growth of yeasts from industrial wastes was inhibited by 50% by high concentrations of Cr(VI): *Candida* sp. by 4 mM Cr(VI) and *Rhodosporidium* sp. by 10 mM Cr(VI) in Sabouraud Broth medium. The other Cr(VI)-sensitive yeasts were inhibited by 0.1 mM Cr(VI). The general mechanism of chromium resistance in *Candida* sp. and *Rhodosporidium* sp. was due to reduced uptake of chromium, but not to biological reduction from Cr(VI) to Cr(III). In Cr(VI)-sensitive yeasts, chromium was accumulated as much as 10-fold, as in *Saccharomyces cerevisiae*. Cr(VI) toxicity in *Candida* sp. was modulated from Cr(VI)-resistance to Cr(VI)-hypersensitivity depending on the addition of methionine, cysteine, sulfate and djenkolic acid. If *Candida* sp. was grown in the presence of S-amino acids, especially methionine, it was more resistant than if the sulfur source was sulfate. When sulfate transport was enhanced by addition of djenkolic acid, *Candida* sp. became hypersensitive. *Rhosporidium* sp. was always resistant to Cr(VI) because sulfate transport was inefficient and it assimilated sulfur as S-amino acids. Cr(VI)-sensitive yeasts required larger amounts of S-amino acids, especially methionine, to tolerate Cr(VI) toxicity. Cysteine was toxic for *C. famata* 6016 above 50 μM,

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Introduction

Chromium is a widespread environmental contaminant which occurs in two main forms: Cr(VI) and Cr(III). The hexavalent form is the more toxic, inhibiting the growth of microorganisms (Aislabie & Loutit 1984), whereas the trivalent form can be tolerated at higher concentrations.

Microorganisms may also show high tolerance to Cr(VI) and it has recently been demonstrated that Cr(VI) can be used as an electron acceptor by a strain of *Enterobacter cloacae* under anaerobic conditions (Wang *et al.* 1989). To date, studies on Cr(VI) resistance have mainly concerned bacteria. Different mechanisms of specific and aspecific chromium-resistance in bacteria have been described (Smillie *et al.* 1981, Bopp *et al.* 1983, Kvasnikof 1983, Ohtake *et al.* 1987, Ishibashi *et al.* 1990) and involve two main mechanisms: Cr(VI) reduction to Cr(III) and reduced uptake of chromium. Similar investigations in eukaryotic cells have been scarce. Chromium-resistance in such cells was first described by Marzluf (1970) in UV-irradiated spores of fungi. In a recent report (Baldi *et al.* 1989), a strain of *Candida* sp. isolated from tannery wastes was demonstrated to be resistant to high concentrations of chromate (4 mM) by virtue of reduced uptake of chromium. This metal is very toxic to yeasts at concentrations of 0.1 mM (Baldi & Pepi 1991) and can induce genotoxic activity by *petite* mutations, as in *Saccharomyces cerevisiae* (Henderson 1989).

The relation between sulfate transport and chromate resistance in *Neurospora crassa* mutants was correlated with partially defective sulfate transport, especially during the conidial stage (Marzluf 1970). In *Pseudomonas fluorescens* strain LB300, the reduced uptake of chromium was also related to the sulfate transport system (Ohtake *et al.* 1987). In this

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case Cr(VI)-resistance was harbored in the plasmid pLHB1. The investigation was carried out in yeasts by adding inhibitor (cysteine) and derepressant (djenkolic acid) of sulfate uptake. In *Anabaena doliolum*, a N_2-fixing cyanobacteria, chromium toxicity was mitigated by the addition of S-amino acids and thiols to culture media (Dubey & Rai 1989), after which nitrogenase activity and heterocyst frequency was restored.

The aim of the present research was to investigate the mechanism of Cr(VI) toxicity in relation to sulfate transport in yeasts isolated from tannery wastes, a *Candida* sp. strain previously described (Baldi *et al.* 1990), and in a *Rhodosporidium* sp. strain isolated from metallurgical industry wastes. A comparison was made with chromium-sensitive strains with similar physiological and biochemical characteristics. The mechanism of Cr(VI) resistance was investigated in relation to chromium uptake with sulfate and S-amino acid additions to cultures stimulating or inhibiting sulfur demand under different culture conditions. Chromium resistance in yeasts is a very new area of study, and should supply information on the differences in metal resistance mechanisms between bacteria and eukaryotic cells.

Materials and methods

Yeast isolation and culturing

Sludge samples were collected in a sterile manner from tannery and metallurgical industry wastes by whirl-pack (Nasco). Microorganisms were isolated in the laboratory on the day of sampling. An aliquot of 0.1 ml from serial dilutions (1:10) of diluted samples was spread on ferropeptone agar plates containing (per liter): polypeptone (Difco), 0.5 g D-glucose, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $(NH_4)_2SO_4$ and 15 g bactoagar (Difco). To isolate Cr(VI)-resistant strains, different concentrations (0.2, 0.5, 1.0, 2.0, 5.0 and 10 mM) of $K_2Cr_2O_7$ were added to the medium. The plates spread with samples were incubated at 28 °C and the colonies counted after 2 and 7 days. The colonies, which differed in size, morphology and color, and grew well on agar plates with 5 and 10 mM chromium, were streaked twice for strain isolation. Yeasts were distinguished from bacteria by differential interference contrast microscopy (Axiophot, Zeiss) and were the only microorganisms thriving at high Cr(VI) concentrations.

Two yeasts were chromium-resistant and were classified by Ann Vaughan-Martini as *Candida* sp. (Vaughan *et al.* 1990), isolated from tannery wastes, and *Rhodosporidium* sp. (Vaughan-Martini, personal communication), isolated from metallurgical wastes. Other chromium-sensitive yeasts with physiological and morphological similarities to the chromium-resistant yeasts were compared. The three chromium-sensitive yeasts physiologically and morphologically close to *Candida* sp. were supplied by the Industrial Yeast Collection, Department of Applied Microbiology, Perugia University, Italy: *C. famata* 6016, *C. famata* 6017 and *Pichia guilliermondii* 6572. Another chromium-sensitive strain utilized in our tests was a strain of *S. cerevisiae* of pharmaceutical origin.

Minimum inhibitory concentration (MIC) tests

Cr(VI)-resistance in yeasts was evaluated by MIC tests. Aliquots of 1 ml of overnight culture were inoculated in 99 ml of Sabouraud Broth (Difco) and 10 ml was distributed in 20 ml test tubes with radial cups. The MIC tests were carried out at different Cr(VI) concentrations from 0.2 to 10 mM for chromium-resistant strains and from 0.02 to 4 mM for chromium-sensitive strains. The samples were incubated for 18 h in a rotary drum at 28 °C. The optical density of the cultures was measured at a wavelength of 600 nm by a UV-visible spectrophotometer (Shimadzu).

MIC tests for Cr(VI) toxicity were performed for *Candida* sp. only in the presence of constant concentrations of cysteine and methionine, with 4 mM sulfate in 1% Yeast Nitrogen Base medium without amino acids and with 2% D-glucose and 0.2 mM djenkolic acid. The concentrations of Cr(VI) ranged from 0.001 to 10 mM.

Influence of sulfur species on Cr(VI) tolerance

Tests for the influence of sulfur species on yeast growth were carried out at a constant Cr(VI) concentration of 0.2 mM, whereas the content of cysteine and methionine added to 1% Yeast Nitrogen Base medium (Difco) without amino acids plus 2% D-glucose varied from 0.0001 to 0.5 mM and from 0.0001 to 10 mM, respectively.

To test for the influence of sulfate on Cr(VI) tolerance, a study was carried out for Cr(VI)-resistant and -sensitive yeasts in Yeast Nitrogen Base medium without amino acids and with NH_4Cl as the nitrogen source. The original $(NH_4)_2SO_4$ was replaced in order to achieve the minimum concentration of total sulfates (4 mM) in the medium. The normal sulfate content in Yeast Nitrogen Base medium is 160 mM.

Chromium accumulation studies

Actively growing cultures of Cr(VI)-resistant yeast strains, from industrial collection, and of *S. cerevisae* in Sabouraud medium were washed twice (centrifugation at 4000 r.p.m. for 20 min) in 10 mM PIPES [piperazine-*N,N*-bis(2-ethanesulfonic acid)] buffer (pH 7.2). The cell suspension was added to a 250 ml conical flask containing 20 ml PIPES buffer with a final concentration of 0.2 mM Cr(VI) plus 1% D-glucose (w/v) to give a final cell concentration of 0.8 ± 0.031 mg ml^{-1} dry weight.

The cells were incubated at 28 °C in a rotary shaking bath at 200 r.p.m. After 18 h of incubation of various concentrations of Cr(VI), 10 ml of sample was removed, centrifuged and washed twice with chromium-free PIPES (centrifugation at 12000 × *g* for 3 min). The washed pellet was mineralized at 60 °C in 0.5 ml concentrated HNO_3 for 1 h, and then cooled and made up with double distilled

water (DDW) to a final volume of 10 ml. The solution was analyzed for total chromium by atomic absorption spectrophotometry (AAS, model 2028 Perkin Elmer) with a graphite furnace (GF, model HGA 5000, Perkin Elmer) and a chromium hollow cathode lamp. Standard additions of chromium were made to the sample for mineralization and calibration. The coefficient of variation of five replicates was 5.2%.

Cr(VI) uptake by the Cr(VI)-tolerant strain of *Candida* sp. was also investigated in the presence of different sources and concentrations of sulfur: species from 4 (minimum) to 30 mM sodium sulfate, from 0.01 to 0.10 mM cysteine and from 0.01 to 0.2 mM djenkolic acid.

Chromium reduction assay

Cells of yeasts were grown in Sabouraud Broth until the mid-logarithmic phase, centrifuged (4000 × *g* for 20 min), and washed twice in 10 mM PIPES and resuspended in PIPES buffer containing 1% (w/v) D-glucose and 0.2 mM Cr(VI). The absorbance of the culture supernatant was measured directly by UV-visible spectrophotometry. Cr(VI) species in aqueous solution absorb at 340–360 nm, whereas Cr(III) species do not.

Results and discussion

The *Candida* sp. strain isolated from tannery wastes has already been described in relation to morphology, physiology and Cr(VI) resistance (Baldi *et al.* 1990). The *Rhodosporidium* sp. strain was classified for yeast identification by Ann Vaughan-Martini by the Van der Walt & Yarrow (1984) methodology.

The *Candida* sp. strain was compared for Cr(VI) resistance with three strains having similar morphological and physiological characteristics, with a chromium-resistant *Rhodosporidium* sp. strain from metallurgical wastes, and with the most common yeast species, *S. cerevisiae*.

The MIC test (Figure 1) showed that the two strains from industrial wastes tolerated high concentrations of Cr(VI). The MIC for *Candida* sp. isolated from tannery wastes was 0.5 mM Cr(VI), while the *Rhodosporidium* sp. from metallurgical wastes was resistant to 8 mM Cr(VI). The MICs for the other strains ranged from 0.1 to 0.05 mM Cr(VI). Despite the complex Sabouraud medium, which probably decreased Cr(VI) toxicity, differences between Cr(VI)-sensitive and -resistant yeasts were very significant.

A test for Cr(VI) reduction to Cr(III) was carried out in all strains after 3 days of incubation at 28 °C. Cr(VI) was not significantly reduced by chromium-sensitive and -resistant strains as compared with the uninoculated samples.

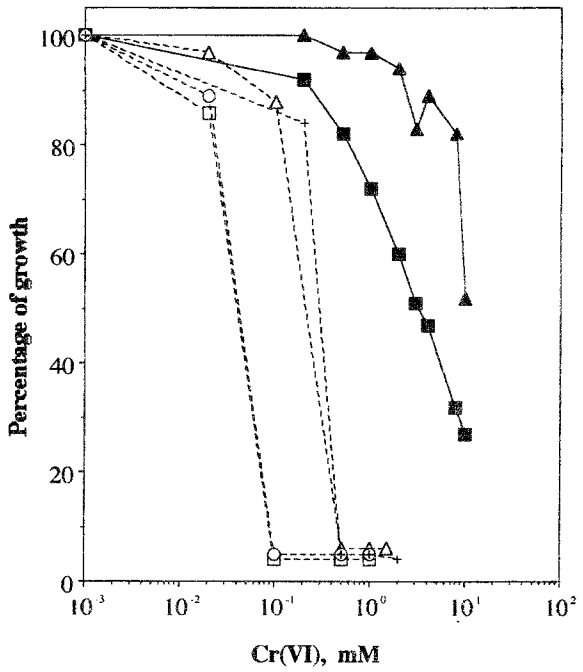

Figure 1. MIC test for *Rhodosporidium* sp. (—▲—), *Candida* sp. (—■—), *Pichia guilliermondii* 6572 (- -+- -), *S. cerevisiae* (- -△- -), *C. famata* 6017 (- -○- -) and *C. famata* 6016 (- -□- -), using a range of Cr(VI) concentrations.

Differences between Cr(VI)-sensitive and -resistant strains were detected by chromium accumulation (Figure 2). Two yeasts, the Cr(VI)-sensitive *C. famata* 6016 and *S. cerevisiae* (especially the latter), accumulated up to 10 times more chromate than *Candida* sp. and *Rhodosporidium* sp. after 18 h of incubation at 28 °C, and at different concentrations from 0.5 (0.01) to 10 $\mu g\,ml^{-1}$ (0.2 mM) of Cr(VI). Reduced chromium uptake is one of the mechanisms utilized by bacteria for tolerating high concentrations of Cr(VI). This mechanism was reported more than two decades ago in UV mutants of *Neurospora crassa* (Marzluf 1970) and more recently in *P. fluorescens* strain LB300 (Ohtake *et al.* 1987). In both cases, Cr(VI) tolerance was related to competition of the toxic oxyanion ($Cr_2O_7^{2-}$) with the essential oxyanion sulfate.

Rhodosporiudium sp. was 2.5 times more resistant than *Candida* sp. but required sulfur in the form of S-amino acids in order to grow. Sulfate was definitely not a sulfur source for this strain of *Rhodosporidium* sp. Cr(VI) resistance was constitutional as in *Candida* sp. (Baldi *et al.* 1990).

The growth plots of two chromium-resistant and two chromium-sensitive yeasts, in the presence of a constant concentration of 0.2 mM Cr(VI) and variable concentrations of cysteine and methionine and

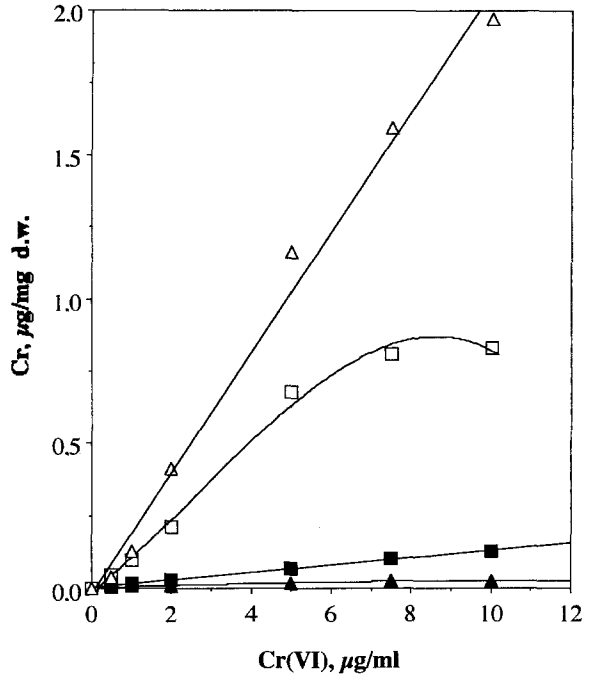

Figure 2. Cr(VI) uptake by yeast cells of *Rhodosporidium* sp. (▲), *Candida* sp. (■), *C. famata* 6016 (□) and *S. cerevisiae* (△) in minimal medium (PIPES + 1% D-glucose) after 18 h of incubation at 28 °C in a rotary shaking bath (200 r.p.m.)

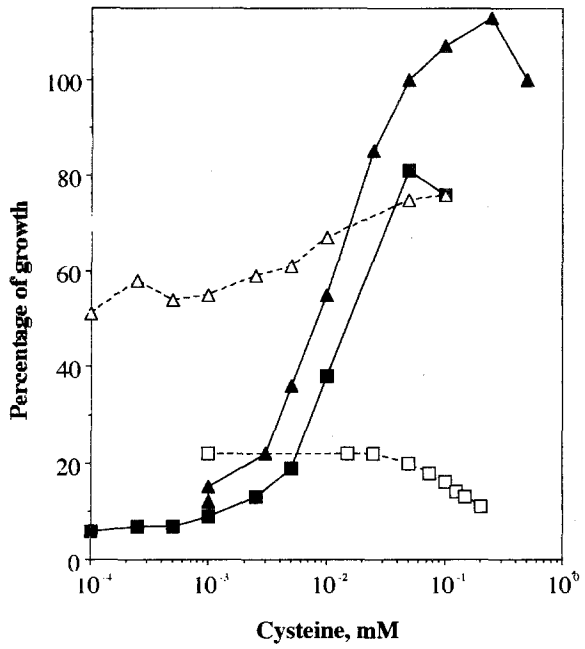

Figure 3. Percentage growth of *Rhodosporidium* sp. (—▲—), *Candida* sp. (—■—), *S. cerevisiae* (- -△- -) and *C. famata* 6016 (- -□- -) in the presence of 0.2 mM Cr(VI) and concentrations of cysteine from 0.0001 to 0.5 mM, incubated at 28 °C.

sulfate, were related to their respective concentrations. Low concentrations (10–100 μM) of cysteine added to the minimal medium significantly increased the optical density of *Rhodosporidium* sp. (10–100% of growth) and almost up to 80% of growth in *Candida* sp. with 100 μM of cysteine (Figure 3). For the two chromium-sensitive species, at higher cysteine contents up to 100 μM, growth only improved by 15% in *S. cerevisiae* and growth inhibition was observed in *C. famata* 6016 above 50 μM of cysteine.

Very low concentrations of methionine (up to 10 μM) were enough to obtain maximum growth in *Rhodosporidium* sp. and 80% in *Candida* sp. The growth of two Cr(VI)-sensitive yeasts also improved in the presence of 0.2 mM Cr(VI), although higher concentrations (0.1–10 mM) of methionine were needed (Figure 4).

The effect of sulfate addition on Cr(VI) tolerance in minimal medium above 4 mM, and at 0.2 mM Cr(VI) concentration, was not evident (Figure 5a) in *Rhodosporidium* sp. or in the two Cr(VI)-sensitive yeasts because (i) *C. famata* 6016 and *S. cerevisiae* should probably be inoculated in a medium with lower concentrations of sulfate, and (ii) the strain of *Rhodosporidium* sp. did not grow because it has a

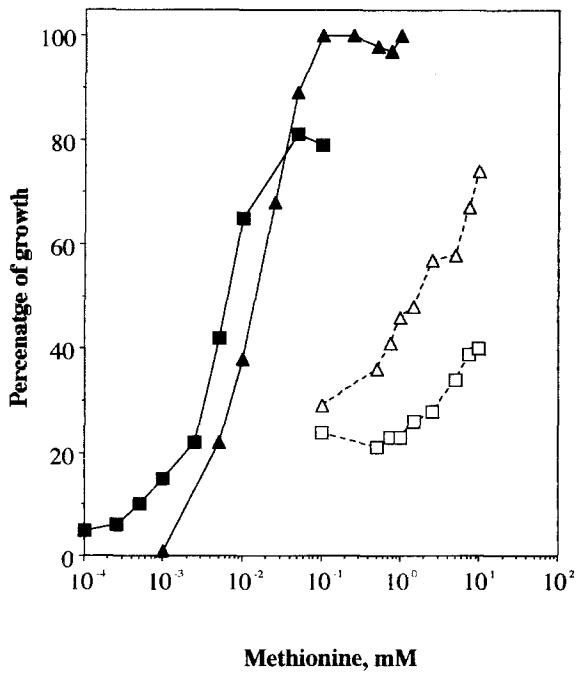

Figure 4. Percentage growth for *Rhodosporidium* sp. (—▲—), *Candida* sp. (—■—), *S. cerevisiae* (- -△- -) and *C. famata* 6016 (- -□- -) in the presence of 0.2 mM Cr(VI) and concentrations of methionine from 0.0001 to 10 mM.

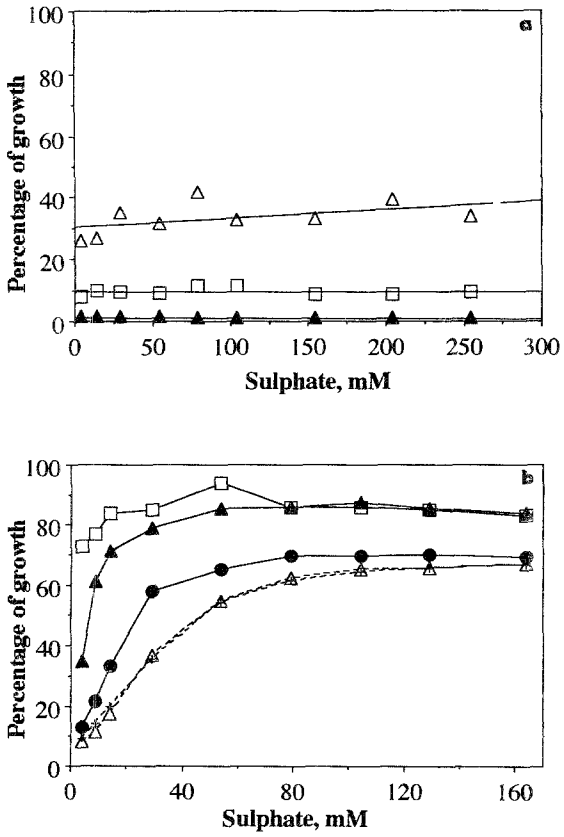

Figure 5. (**a**) Percentage growth of *Rhodosporidium* sp. (—▲—), *S. cerevisiae* (—△—) and *C. famata* 6016 (—□—) in the presence of 0.2 mM Cr(VI) and variable concentrations of sulfate from 4 to 250 mM. (**b**) Percentage growth of *Candida* sp. with five different concentrations of Cr(VI), i.e. 0.04 mM (—□—), 0.08 mM (—▲—), 0.12 mM (—●—), 0.16 mM (- -△- -) and 0.2 mM (- -+- -), and variable concentrations of sulfates from 4 to 160 mM.

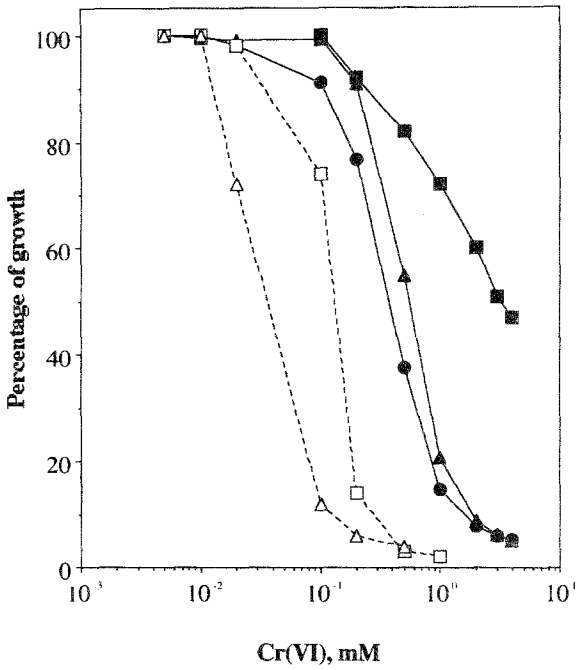

Figure 6. MIC test for *Candida* sp. in Sabouraud medium (—■—) and in Yeast Nitrogen Base plus 2% D-glucose with 0.04 mM methionine (—▲—), 0.08 mM cysteine (—●—), 4 mM sulphate (- -□- -) and 0.2 mM djenklic acid (- -△- -), and variable concentrations of Cr(VI) from 0.001 to 10 mM.

defective sulfate transport system. *Candida* sp., however, tolerated Cr(VI) toxicity in relation to sulfate spikes (Figure 5b). The maximum growth of *Candida* sp. was observed in Yeast Nitrogen Base medium with 2 and 4 $\mu g\,ml^{-1}$ chromium in the presence of 50 mM sulfate.

The MICs for *Candida* sp. depended on the sulfur species in the minimal medium (Figure 6). The highest Cr(VI) tolerance (MIC = 0.5 mM) was observed with a medium with 40 μM of methionine and in the complex medium Sabauroud. *Candida* sp. became less chromium-tolerant with 80 μM of cysteine (MIC = 0.2 mM) and even less tolerant with 4 mM of sulfate (MIC = 0.1 mM), like *C. famata* 6016 and *S. cerevisiae* inoculated in the complex medium Sabauroud. The strain of *Candida* sp. became hypersensitive (MIC = 0.02 mM) when the same minimal medium was spiked with 0.2 mM of djenkolic acid, a S-amino acid of plant origin, which derepresses the microbial sulfate transport system (Ohtake *et al.* 1987).

Chromium accumulation experiments confirmed that Cr(VI) resistance and sensitivity in *Candida* sp. was modulated and was related to chromium uptake by yeast cells. The experiments were carried out with a constant concentration of Cr(VI) (6 $\mu g\,ml^{-1}$, 0.12 mM), and with variable sulfate and S-amino acid (cysteine and djenkolic acid) contents. The concentrations were varied and were chosen from MIC tests. The linear decrease in total chromium in *Candida* sp. cells showed that sulfate uptake prevented Cr(VI) uptake (Figure 6a). Cysteine inhibited sulfate transport and consequently reduced chromium uptake in *Candida* sp. (Figure 6b). At higher concentrations of cysteine (up to 0.1 mM) a toxic effect of this amino acid probably occurred, as observed for *C. famata* 6016 (Figure 3). The effect of djenkolic acid (0.2 mM) (Figure 6b) was to significantly enhance total chromium concentrations in *Candida* sp. cells.

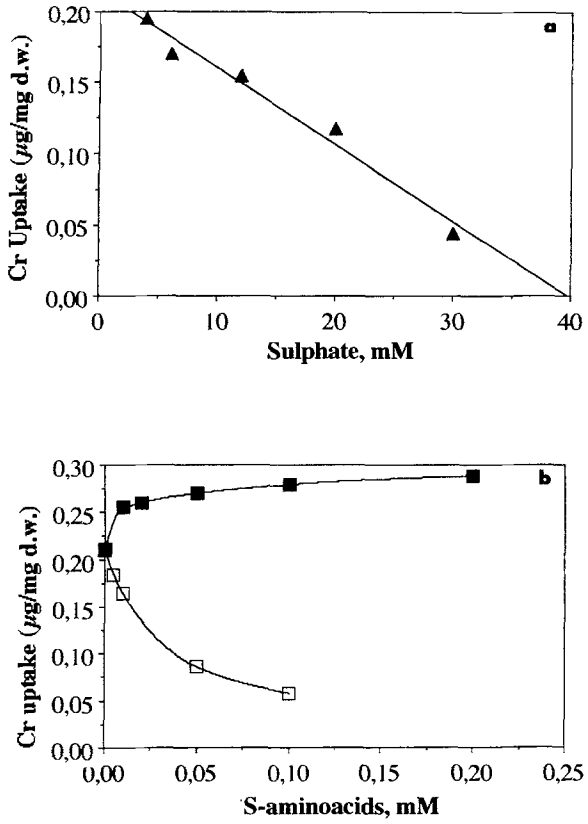

Figure 7. (**a**) Linear decrease in total chromium (μg mg^{-1} dry weight) uptake in *Candida* sp. (—□—) after sulfate additions from 4 to 30 mM to Yeast Nitrogen Base medium plus 2% D-glucose, incubated for 18 h at 28 °C. (**b**) Decrease in total chromium uptake after cysteine additions (—△—) from 0.005 to 0.1 mM in *Candida* sp. and chromium uptake enhancement after additions of djenkolic acid (—▲—) from 0.01 to 0.2 mM under the same culturing conditions.

Conclusion

Cr(VI)-resistant yeasts did not reduce Cr(VI) to Cr(III). Their Cr(VI)-tolerance mechanism is in some ways similar to that of certain bacteria. In *P. fluorescens*, Othake *et al.* (1987) observed that reduced accumulation of chromium depended on sulfate transport. This system is the preferential mechanism by which chromates pass actively through the cell envelope; however, Cr(VI) tolerance is affected by other sulfur species, like cysteine, an inhibitor of sulfate transport. Cysteine enhances Cr(VI) tolerance in bacteria and yeasts.

Rhodosporidium sp., which does not grow when sulfate is the sole sulfur source, showed constitutional resistance to Cr(VI); likewise *Candida* sp., but they have a slightly different mechanism of Cr(VI) resistance. The *Rhodosporidium* sp. was always more resistant than *Candida* sp., and the Cr(VI) resistance in *Rhodosporidium* sp. did not depend on sulfur species except as a growth factor. Cr(VI) tolerance in *Candida* sp., growing in minimal medium (Yeast Nitrogen Base), was modulated by the presence of different sulfur species, in a manner intermediate between the chromium-resistant *Rhodosporidium* sp. and the Cr(VI)-sensitive yeasts. In the presence of S-amino acids (methionine and cysteine), especially methionine, *Candida* sp. was almost as Cr(VI)-resistant as in the complex Sabouraud medium. In the presence of different concentrations of sulfates, as the sole sulfur source, *Candida* sp. tolerated Cr(VI) toxicity less, although a significant increase in resistance to 0.12 mM Cr(VI) was observed in relation to sulfate additions. In the presence of djenkolic acid, a sulfate derepressant S-amino acid, *Candida* sp. tolerated very low concentrations of Cr(VI) and assimilated chromium intracellularly, whereas the *Rhodosporidium* sp. was always Cr(VI)-resistant under these different conditions.

The other Cr(VI)-sensitive yeasts, *S. cerevisiae* and *C. famata* 6016, required a huge amount of S-amino acids to tolerate Cr(VI) toxicity compared with the two Cr(VI)-resistant yeasts, i.e. *Rhodosporidium* sp. and *Candida* sp. Cysteine was shown to be toxic for *C. famata* 6016 even at a concentration above 50 μM. Methionine was originally considered the best compound for mitigating Cr(VI) toxicity in Cr(VI)-resistant and Cr(VI)-sensitive yeasts. In *A. doliolum*, addition of cysteine and methionine and other thiols (mercaptoethanol, dithiothreitol) restored cyanobacterium growth and nitrogen fixation in the following order of protective efficiency: cysteine > methionine > thiols (Dubey & Rai 1989). In our yeasts, the order was methionine > cysteine > sulfate.

Sulfates play an important role in Cr(VI) tolerance, as shown for *Candida* sp., but the sulfate transport system probably functions at lower Cr(VI) concentrations in Cr(VI)-sensitive yeasts. This threshold could not be investigated further because of our methodology (no radioactive compounds were used) and, besides, the sulfate concentration in the media was at least 4 mM. In Cr(VI)-sensitive strains, a competitive behavior, similar to that of *Candida* sp., but at lower sulfate and chromate concentrations, could probably be explored.

Acknowledgments

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