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Chromate tolerance in strains of *Rhodosporidium toruloides* modulated by thiosulfate and sulfur amino acids

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Cr(VI) tolerance was studied in four strains of *Rhodosporidium toruloides* and compared with that of a fifth strain, DBVPG 6662, isolated from metallurgical wastes and known to be Cr(VI) resistant. Tolerance was studied in relation to different species of sulfur (sulfates, thiosulfates, methionine, cysteine) at different concentrations. Djenkolic acid, a poor source of sulfur and an activator of sulfate transport, was also considered. In synthetic medium all strains except the Cr(VI)-resistant one started to be inhibited by 10 $\mu g\,ml^{-1}$ (0.2 mM) Cr(VI) as $K_2Cr_2O_7$. DBVPG 6662 was inhibited by 100 $\mu g\,ml^{-1}$ (2.0 mM) Cr(VI). In Yeast Nitrogen Base without amino acids (minimal medium), supplemented with varying concentrations of chromate, all Cr(VI)-sensitive strains accumulated concentrations of total chromium (from 0.8 to 1.0 $\mu g\,mg^{-1}$ cell dry wt) after 18 h of incubation at 28 °C. In minimal medium supplemented with 10 $\mu g\,ml^{-1}$ Cr(VI), the addition of sulfate did not significantly improve the yeast growth. Cysteine at μM levels increased tolerance up to 10 $\mu g\,ml^{-1}$, whereas methionine only reduced the Cr(VI) toxicity in the strain DBVPG 6739. Additions of djenkolic acid resulted in increased Cr(VI) sensitivity in all strains. The best inorganic sulfur species for conferring high tolerance was thiosulfate at concentrations up to 1 mM. In all cases increased Cr(VI) tolerance was due to a significantly reduced uptake in the oxyanion by the cells and not to the chemical reduction of Cr(VI) to Cr(III) by sulfur compounds.

Keywords: yeasts, *Rhodosporidium toruloides*, chromate, sulphates, thiosulphate, cysteine, methionine, djenkolic acid, tolerance, Cr uptake

Introduction

The genetic and physiological mechanisms of cell protection against metal ions have been extensively studied and were recently reviewed by Silver & Walderhaug (1992). Chromate resistance in the bacteria *Enterobacter cloacae* (Wang *et al.* 1989), *Pseudomonas putida* (Ishibashi *et al.* 1990) and recently in *Pseudomonas ambigua* (Suzuki *et al.* 1992), has been investigated mainly in relation to Cr(VI) reduction to Cr(III), since this could have biotechnological applications in the treatment of industrial wastes. A second mechanism, described in bacterial cells, is a plasmid-determined resistance due to reduced accumulation of Cr(VI) by cells of *Pseudomonas fluorescens* (Summers & Jacoby 1978, Ohtake *et al.* 1987, Cervantes & Ohtake 1988). This has also been demonstrated in species of other genera such as *Alcaligenes* (Nies *et al.* 1989) and *Streptococcus* (Efstatiou & McKay 1977).

It has been clearly shown that the sulfate transport system has a high affinity for chromate as an alternative substrate and that mutations in the system significantly affect its uptake (Marzluf 1970, Ohtake *et al.* 1987). However, the uptake transport pathway is not directly involved since the kinetic parameters for the uptake of sulfate are unaltered by the chromate resistance plasmid (Silver & Walderhaug 1992). The physiological roles of two genes related to Cr(VI) resistance (*ChrA* and *ChrB*) are still under investigation.

In eukaryotic cells chromate resistance depends on reduced uptake of Cr(VI). Marzluf (1970) selected UV-induced mutant spores of *Neurospora crassa* with Cr(VI) resistance and found that all of them had partially defective sulfate transport systems, especially during the conidial stage. Two different sulfate channels, identified as permeases I and II, the latter produced in the mycelial phase, were repressed by methionine and acted as corepressors of enzymes such as aryl-sulfates, choline sulfatase

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choline-O-sulfate permease and sulfate permease (Metzenberg & Parson 1966).

A yeast isolated from tannery wastes and identified as a species of the genus *Candida* was found to be Cr(VI) resistant in the presence of cysteine and methionine. It was found that inhibited sulfate transport was responsible for the reduced uptake of chromium in this strain (Baldi *et al.* 1990). In contrast, in Yeast Nitrogen Base medium without amino acids, supplemented with sulfate, the strain became as sensitive to the oxyanion as other strains of *Candida* tested. In the presence of djenkolic acid, a derepressant of sulfate transport, Cr uptake was enhanced and the strain became more sensitive to chromate (Pepi & Baldi 1992). Another yeast, isolated from metallurgical wastes and identified as a *Rhodosporidium toruloides*, was found to be highly Cr(VI) resistant as a result of an inefficient sulfate transport system. This yeast can utilize cysteine, methionine or thiosulfate, but not sulfate, as sole sulfur sources (Pepi & Baldi 1992).

The aim of this study was to investigate the different responses to chromate of further strains of the species *R. toruloides* and to test whether sensitive strains become more tolerant to chromate in the presence of S-amino acids and/or other sulfur species. We also investigated whether the Cr(VI) tolerance found in some strains of *R. toruloides* was linked not to sulfates but to another inorganic sulfur species, thiosulfate.

Materials and methods

Microorganism culture conditions

Four strains of the species *R. toruloides* were obtained from the Industrial Yeasts Collection of the Dipartimento di Biologia Vegetale (DBVPG), Università di Perugia, Perugia, Italy and were designated DBVPG 6739, DBVPG 6740, DBVPG 6742 and DBVPG 6743. A fifth, Cr(VI)-resistant strain was used as control for the Cr(VI) tolerance tests. This strain, previously isolated from metallurgical industry wastes (Pepi & Baldi 1992), was identified at the DBVPG as belonging to the species *R. toruloides* on the basis of classical phenotypic tests as well as nDNA/nDNA reassociation with the type strain of that epithet, and was designed DBVPG 6662. All strains were maintained on Sabouraud broth (Difco) and grown on YEPG medium containing 10 g yeast extract, 10 g peptone and 20 g D-glucose per l.

Minimum inhibitory concentration tests

The five strains of yeast were tested for growth in the presence of different concentrations of Cr(VI). This test was performed in a minimal medium consisting of 2 g Yeast Nitrogen Base (Difco), 2 g D-glucose and 0.6 g NH_4Cl per l. Only the strain *R. toruloides* DBVPG 6662 was grown in minimal medium with 0.1 mM of cysteine (Carlo Erba) as sulphur source. Aliquots of 1 ml of overnight cultures were inoculated in 99 ml of minimal medium and 10 ml of this subsample was distributed in 20 ml test tubes with radial cups. Cr(VI) concentrations (0.2–10 mM for DBVPG 6662 and 0.02–2.0 mM for the other four strains) were added to each test tube and the inoculated test tubes were incubated for 18 h at 28 °C and aerated in a rotary drum. Yeast growth was measured in terms of absorbance at 600 nm, using a UV-visible spectrophotometer (Shimadzu 160 S).

Influence of sulphur species on Cr(VI)-tolerance

Tests of growth in the presence of a constant Cr(VI) concentration (0.2 mM = 10 $\mu g\,ml^{-1}$) and different concentrations of five different sources of sulfur were carried out for the Cr(VI)-resistant and Cr(VI)-sensitive yeast strains. Aliquots of 1 ml of overnight cultures were inoculated in 99 ml of minimal medium. Ten ml of this subsample was distributed in 20 ml test tubes with radial cups, and different concentrations of cysteine (0.0025–0.5 mM), methionine (0.01–1.0 mM), thiosulfate (0.15–6.25 mM) and djenkolic acid (0.005–0.25 mM) were added. The test tubes were incubated at 28 °C for 18 h in a rotary drum and growth measured on the basis of optical density at 600 nm.

Chromium uptake

Cr uptake was determined in the Cr(VI)-sensitive strains DBVPG 6739, 6742, 6743 and in the Cr(VI)-resistant strain 6662 in the presence of different concentrations of Cr(VI) from 0.01 mM to 0.1 mM (0.5–5 $\mu g\,ml^{-1}$). Cells in the exponential phase of growth were harvested by centrifugation at 1480 × *g* for 20 min and washed twice with 10 mM PIPES (piperazine-*N*,*N*′-bis(2-ethansulfonic-acid)) buffererd at pH 7.4. The pellet was placed in a 250 ml conical flask containing 150 ml PIPES and 1% D-glucose. Twenty ml of this suspension was added to 100 ml conical flasks and different spiked concentrations of Cr(VI) ranging from 0.25 mM to 5.0 mM. The inoculated conical flasks were incubated at 28 °C in a rotary shaking bath at 250 r.p.m. After 18 h, 10 ml of sample was removed, centrifuged at 1480 × *g* for 20 min. The pellet was washed twice with PIPES buffer and mineralized at 60 °C for 1 h with 1 ml concentrated HNO_3. When the solution had cooled, it was made up to a final volume of 10 ml with double distilled water. The solution was analyzed for total chromium by atomic absorption spectrophotometry (AAS, model 2028 Perkin Elmer) using a graphite furnace (GF, Model HGA 5000, Perkin Elmer) equipped with a hollow cathode lamp for chromium. Standard additions of chromium were made to the sample for mineralization and calibration. The coefficient of variation of five replicates was 5.2%.

Chromium uptake in the presence of sulphur species

The Cr(VI)-sensitive strains DBVPG 6739, 6742 and 6743 were analyzed for chromium uptake in the presence of thiosulfate (0.15–6.25 mM) and in the presence of djenkolic acid (0.0025–0.2 mM). The tests were carried out at a constant Cr(VI) concentration of 0.2 mM. The procedure for Cr uptake and determination was as above.

Chromium reduction assay

Yeasts were grown in Sabouraud medium up to mid-logarithmic phase, centrifuged at 1480 × *g* for 20 min and washed twice in 10 mM PIPES. They were resuspended in Yeast Nitrogen Base medium plus 1% D-glucose amended with 0.2 mM Cr(VI) plus the respective sulfur sources, to determine their chemical and biological reducing activity towards Cr(VI). The absorbance of the culture supernatant was measured directly by UV–visible spectrophometry. The Cr(VI) oxyanion in aqueous solution absorbs at 340–360 nm whereas Cr(III) does not.

Results and discussion

The growth of five strains of *R. toruloides* in the presence of different concentration of chromates is shown in Figure 1. However, even if dichromate ($Cr_2O_7^{2-}$) is added to the medium there is an equilibrium resulting in the chromate ($Cr_2O_4^{2-}$) ion. The equilibrium in diluted solutions is toward chromate ion formation according to the equation:

$$2CrO_4^{2-} + 2H^+ \rightleftharpoons Cr_2O_7^{2-} + H_2O$$

So, dichromate in aqueous solution is mainly transformed to chromate.

Four strains were inhibited by minimum Cr(VI) concentrations (0.2 mM). This threshold value was higher than for other Cr(VI)-sensitive species of yeasts grown in the same medium at same temperature (Pepi & Baldi 1992). Resistance to Cr(VI) toxicity was thus verified in the species *R. toruloides*. In the DBVPG 6662 strain, Cr(VI) resistance persisted up to 2.0 mM (100 $\mu g\,ml^{-1}$) Cr(VI), and the mechanism was related to an inefficient sulfate transport system. No yeast growth was observed with this sulfur source, even without chromate additions (Figure 2). The uptake of Cr by strain 6662 was consequently negligible (Pepi & Baldi 1992) since chromates pass through the same transport system (Silver & Walderhaug 1992).

In the Cr(VI) uptake study, three Cr(VI)-sensitive strains, DBVPG 6739, 6742 and 6743, accumulated high concentrations of chromium when incubated for 18 h at 28 °C with 0.01–0.1 mM (0.5–5.0 $\mu g\,ml^{-1}$) Cr(VI) (Figure 3). The Cr(VI) sensitivity in the yeasts was due to Cr accumulation, which was highest in strain 6743. As expected, strain DBVPG 6662 did not accumulate Cr. The toxicity of chromate is often related to transport and/or reduction of Cr(VI) to Cr(III) by cells. In our experiments extracellular reduction of Cr(VI) to Cr(III) was not significant, but an intracellular reduction cannot be excluded (De Flora & Wetterhahn 1989).

In a previous study, a Cr(VI)-resistant yeast, identified as *Candida* sp. strain DBVPG 6502, was demonstrated to

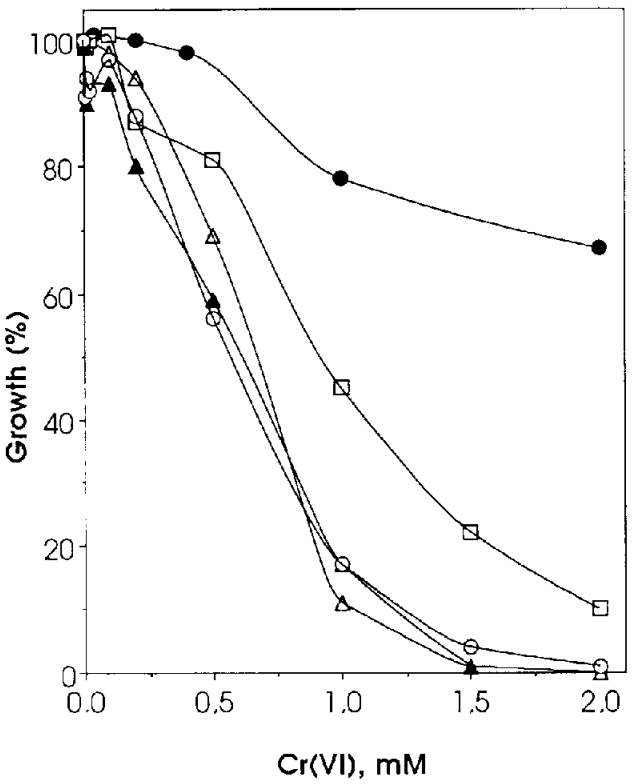

Figure 1. Minimum inhibitory concentration test in *R. toruloides* strains DBVPG 6662 (●), 6739 (△), 6740 (□), 6742 (▲) and 6743 (○), exposed to various concentrations of Cr(VI) for 18 h at 28 °C.

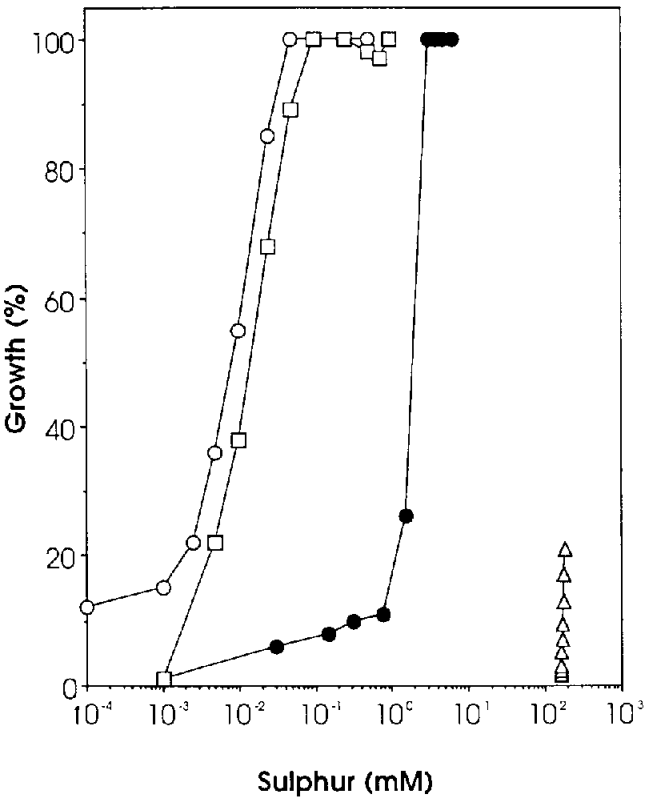

Figure 2. Percentages of growth for *R. toruloides* strain DBVPG 6662 following additions of various concentrations of cysteine (○), methionine (□), thiosulfate (●) and sulfates (△) as sulfur sources in minimal medium.

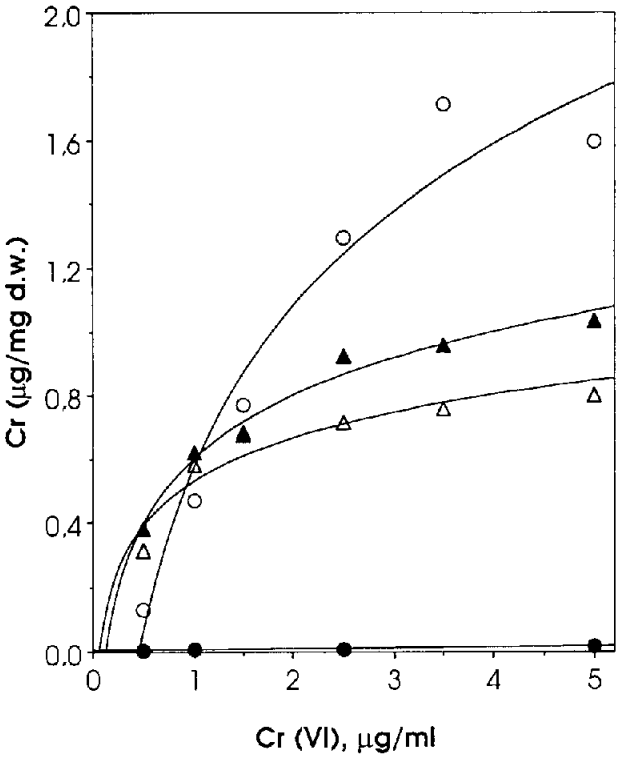

Figure 3. Cr uptake by cells (µg/mg dry weight) in *R. toruloides* strains DBVPG 6662 (●), 6739 (△) 6742 (▲) and 6743 (○), exposed to various concentrations of Cr(VI) in PIPES buffer plus 1% D-glucose for 18 h.

be resistant to Cr(VI). This was due to the presence of sulfur forms such as sulfates, methionine and cysteine. The two amino acids were preferentially used as sulfur source in the presence of chromate. Sulfate transport was turned off and consequently the oxyanion accumulation rate was significantly reduced. The presence of another sulfur amino acid of plant origin, djenkolic acid, stimulated sulfate transport and *Candida* sp. became hypersensitive to Cr(VI) due to an increase in Cr accumulation (Pepi & Baldi 1992).

When a similar study was performed in four strains of *R. toruloides* exposed to 0.2 mM (10 µg ml^{-1}) Cr(VI), in order to investigate Cr(VI) tolerance in relation to different sulfur sources, growth did not improve significantly with increasing concentrations of sulfates. Strains of *Saccharomyces cerevisiae Candida famata* and *Pichia guilliermondii* can tolerate 0.2 mM Cr(VI) when 40–80 mM sulfates are added (Pepi & Baldi 1992). So, in the *R. toruloides* species the transport of sulfate is in general less efficient than that of other sulfur forms. This physiological difference imparts high tolerance to Cr(VI).

All sensitive strains instead became tolerant to 0.2 mM Cr(VI) when 1 mM thiosulfate was added to the medium. The strain 6739 needed less thiosulfate (only 0.3 mM) to become Cr(VI) tolerant. The Cr(VI)-resistant strain 6662 also used this inorganic sulfur source (Figure 4a), but the concentration of thiosulfate required to obtain optimal growth was significantly higher than for other strains of *R. toruloides*, and was ten times higher than for strain 6739.

Three sensitive strains became tolerant to 0.2 mM Cr(VI) when 0.25 mM cysteine was added to the minimal medium (Figure 4b). Cultures of DBVPG 6743 had to be amended with a higher concentration of cysteine (0.5 mM) to obtain optimal growth. The Cr(VI)-resistant strain DBVPG 6662, in contrast, reached maximum growth with one-tenth of the concentration of cysteine (0.05 mM) and methionine (0.1 mM), suggesting that S-amino acid transport in strain 6662 was more efficient than in the other Cr(VI) sensitive strains. This finding also indicates that sulfur amino acids are preferred to thiosulfate, which is the best inorganic sulfur source for yeasts of this species. Different responses to Cr(VI) toxicity were observed when methionine was added to the cultures (Figure 4c). Strain 6739 tolerated 0.2 mM Cr(VI) with 0.75 mM methionine. Higher concentrations of this amino acid slightly improved the growth of strains 6740, 6742 and 6743. This demonstrated that cysteine was the best S-amino acid to reduce Cr(VI) toxicity in *R. toruloides* strains.

When djenkolic acid, an enhancer of sulfate transport, was added, strains 6739 and 6742 became sensitive to 0.2 mM Cr(VI) at djenkolic acid concentrations above 0.1 mM. Strains 6740 and 6743 were more sensitive to the addition of this amino acid (Figure 4d). The Cr(VI)-resistant strain 6662, on the other hand, cannot grow as well with this S-amino acid as a sulfur source as it can in the presence of sulfate.

The increased tolerance of *R. toruloides* to Cr(VI) toxicity may also be ascribed to the chemical reducing power of thiosulfate, cysteine and methionine (De Flora & Wetterhahn 1989). When Cr(VI) reduction was analyzed, differences in chemical reduction were found of around 18.1 ± 4.2% in an 18 h experiment in inoculated and uninoculated samples. Studies with ^{35}S-isotope on competition between sulfate and chromate transport are well documented (Marzluf 1970, Benitez *et al.* 1983, Ohtake *et al.* 1987). In our study we only quantify chromium uptake by cells to demonstrate that chromate resistance is due to the diminished uptake of this metal and not to the biological reduction of Cr(VI) to Cr(III), which was not found in our experiments.

When the strains 6739, 6742 and 6743 were grown with different concentrations of thiosulfate and a constant concentration of chromate (0.2 mM), Cr uptake dropped significantly from strain to strain (Figure 5a). The thiosulfate was very effective in increasing Cr(VI) tolerance in strain DBVPG 6743, and less so in the other two strains. On the other hand, in the presence of djenkolic acid a significant increase in Cr accumulation was observed only in strain DBVPG 6742 (Figure 5b). This indicates that in this strain, unlike in other species of yeasts (Pepi & Baldi 1992), chromate is transported by a sulfate transport system with low affinity. Djenkolic acid facilitates chromium accumulation in prokaryotic and eukaryotic cells with efficient sulfate transport systems (Marzluf 1970, Ohtake *et al.* 1987).

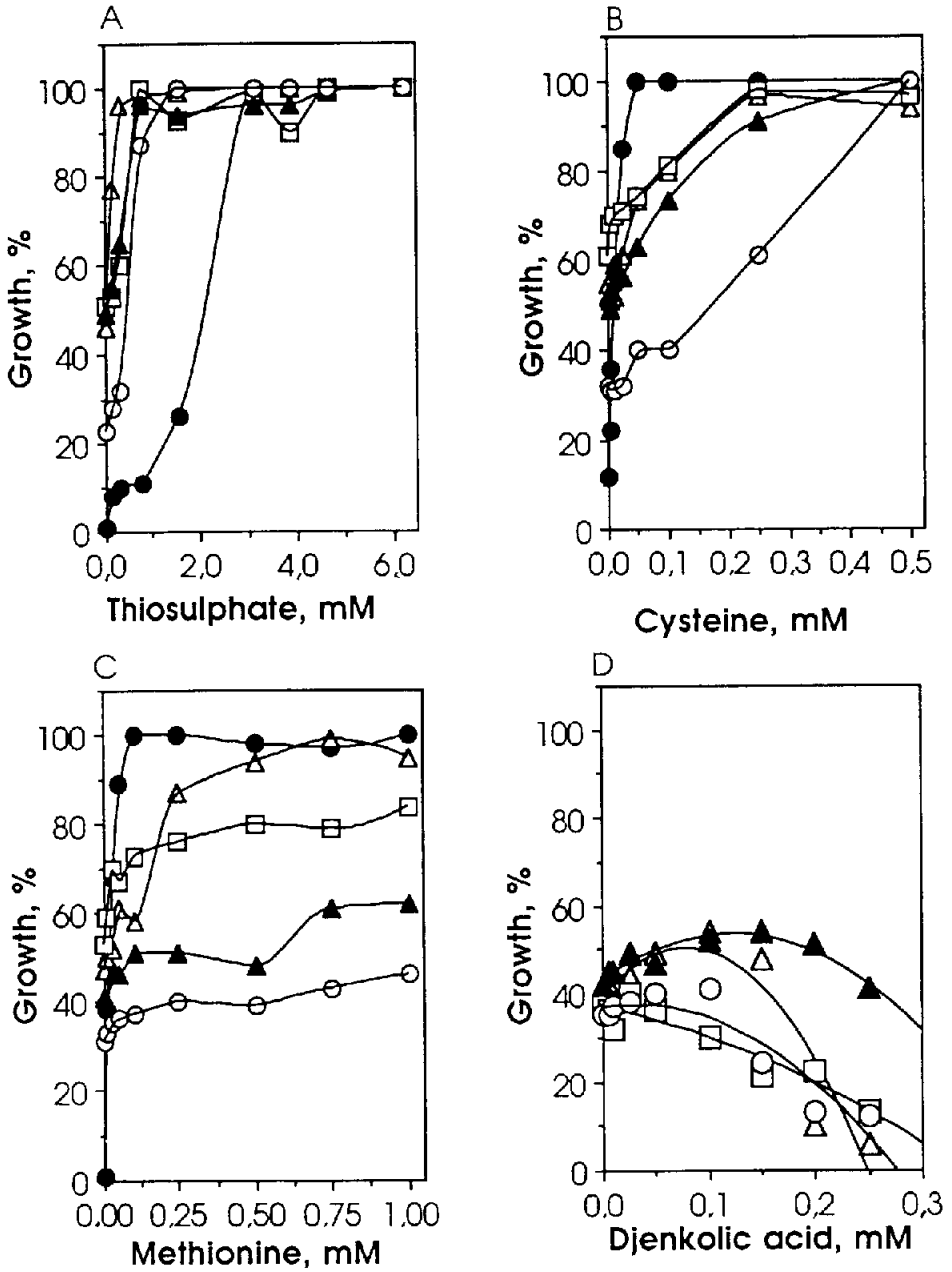

Figure 4. Percentage growth of *R. toruloides* strains DBVPG 6662 (●), 6739 (△), 6740 (□), 6742 (▲) and 6743 (○) in the presence of 0.2 mM chromate and various concentrations (mM) of (**A**) sodium thiosulfate, (**B**) cysteine, (**C**) methionine and (**D**) djenkolic acid.

Cr(VI) tolerance in relation to sulfur species such as thiols and amino acids has also been demonstrated in the cyanobacterium *Anabaena doliolum* (Dubey & Rai 1989). Cysteine at a concentration of 0.05 mM was the best sulfur-containing amino acid for restoring growth, heterocyst differentiation and nitrogen fixation after exposure to high concentrations of Cr(VI). However, in bacteria such as *Pseudomonas fluorescens*, sulfates play a most important role in Cr(VI) tolerance as antagonists for active chromate transport (Ohtake *et al.* 1987). The sulfate transport system has been studied at molecular level in *Escherichia coli* (Sirko *et al.* 1990). The active transport of this nutrient is located in the same gene cluster (*cysTWAM*) as thiosulfate transport. However this sulfur species is scavenged in the periplasmic space by a specific protein, CysP (Hryniewicz *et al.* 1990), and does not bind sulfates, which are scavenged by another periplasmic protein, Sbp (Silver & Walderhaug 1992).

The role of thiosulfate as antagonist of chromate uptake has not yet been investigated. Although sulfur assimilation in prokaryotic and eukaryotic cells cannot rightly be compared, the present results show that thiosulfate is also an important antagonist for chromate uptake. Cysteine has been demonstrated to be the best amino acid for decreasing Cr(VI) toxicity in *R. toruloides*; at higher concentrations the same sulphur species may inhibit the growth of

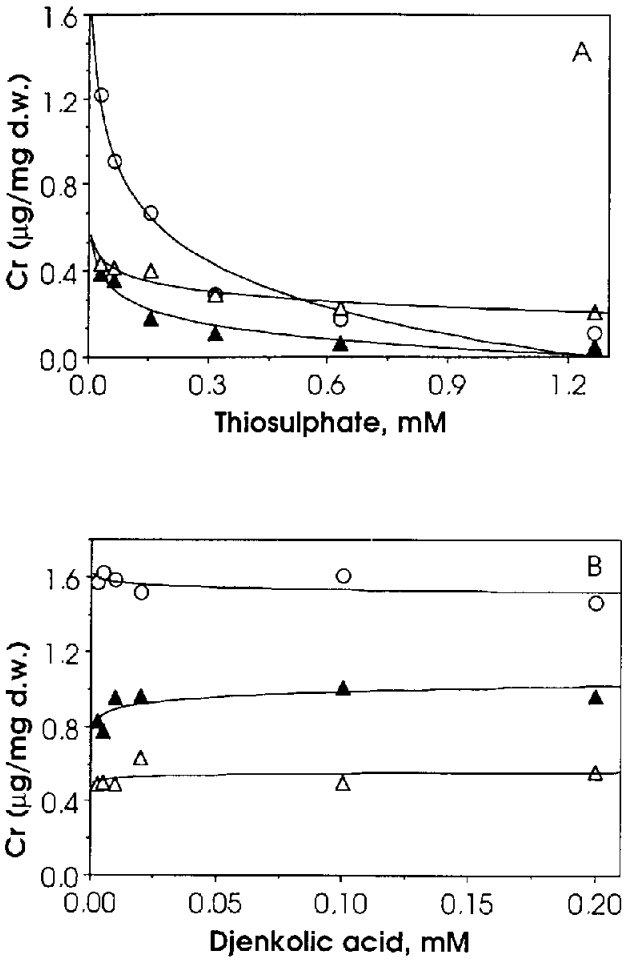

Figure 5. (**A**) Reduced accumulation of Cr in cells of *R. toruloides* strains DBVPG 6739 (△), 6742 (▲) and 6743 (○), in relation to thiosulfate additions. (**B**) Unchanged or enhanced accumulation of total chromium in the same strains in the presence of djenkolic acid.

other yeast species (Pepi & Baldi 1992). Cysteine also improved growth in the presence of chromate in *Anabaena doliolum* (Dubey & Rai 1989). Molecular studies of the sulfate transport system in *R. toruloides* are required for an understanding of the mechanism of this special sulfur demand.

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References

Baldi F, Vaughan Martini AM, Olson GJ. 1990 Chromium(VI)-resistant yeast isolated from a sewage treatment plant receiving tannery wastes. *Appl Environ Microbiol* **56**, 913–918.

Benitez JA, Alonso A, Delgado J, Kotyk A. 1983 Sulphate transport in *Candida utilis*. *Folia Microbiol* **28**, 6–11.

Cervantes C, Ohtake H. 1988 Plasmid-determined resistance to chromate in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* **56**, 173–176.

De Flora S, Wetterhahn KE. 1989 Mechanisms of chromium metabolism and genotoxicity. *Life Chem Rep* **7**, 169–244.

Dubey SK, Rai LC. 1989 Toxicity of chromium and tin to *Anabaena doliolum*. *Biol Metals* **2**, 55–60.

Efstathiou JD, McKay LL. 1977 Inorganic salts resistance associated with a lactose-fermenting plasmid in *Streptococcus lactis*. *J Bacteriol* **130**, 257–265.

Hryniewicz M, Sirko A, Palucha A, Bock A, Hulanicka D. 1990 Sulfate and thiosulfate transport in *Escherichia coli* K-12: identification of a gene encoding a novel protein involved in thiosulfate binding. *J Bacteriol* **172**, 3358–3366.

Ishibashi Y, Cervantes C, Silver S. 1990 Chromium reduction in *Pseudomonas putida*. *Appl Environ Microbiol* **56**, 2268–2270.

Marzluf GA. 1970 Genetic and metabolic controls for sulfate metabolism in *Neurospora crassa*: isolation and study of chromate-resistant and sulfate transport-negative mutants. *J Bacteriol* **102**, 716–721.

Metzenberg RL, Parson JW. 1966 Altered repression of some enzymes of sulfur utilization in a temperature-conditional lethal mutant of *Neurospora*. *Proc Natl Acad Sci USA* **55**, 629–635.

Nies A, Nies DH, Silver S. 1989 Cloning and expression of plasmid genes encoding resistances to chromate and cobalt in *Alcaligenes eutrophus*. *J Bacteriol* **171**, 5065–5070.

Ohtake H, Cervantes C, Silver S. 1987 Decreased chromate uptake in *Pseudomonas fluorescens* carrying a chromate resistance plasmid. *J Bacteriol* **169**, 3853–3856.

Pepi M, Baldi F. 1992 Modulation of chromium(VI) toxicity by organic and inorganic sulfur species in yeasts from industrial wastes. *BioMetals* **5**, 179–185.

Silver S, Walderhaug M. 1992 Gene regulation of plasmid and chromosome-determined inorganic ion transport in bacteria. *Microbiol Rev* **56**, 195–228.

Sirko A, Hryniewcz M, Hulanicka D, Bock A. 1990 Sulfate and thiosulfate transport in *Escherichia coli* K-12: nucleotide sequence and expression of the *cysTWAM* gene cluster. *J Bacteriol* **172**, 3351–3357.

Summers AO, Jacoby GA. 1978 Plasmid-determined resistance to boron and chromium compounds in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **13**, 637–640.

Suzuki T, Miyata N, Horitzu H *et al.* 1992 NAD(P)H-dependent chromium(VI) reductase of *Pseudomonas ambigua* G-1: a Cr(VI) intermediate is formed during the reduction of Cr(VI) to Cr(III). *J Bacteriol* **16**, 5340–5345.

Wang P, Mori T, Komori K *et al.* 1989 Isolation and characterization of an *Enterobacter cloacae* strain that reduces hexavalent chromium under anaerobic conditions. *Appl Environ Microbiol* **55**, 1665–1669.