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Chromium(VI)-Resistant Yeast Isolated from a Sewage Treatment Plant Receiving Tannery Wastes

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A Cr(VI)-resistant yeast, designated strain DBVPG 6502, was isolated from a sewage treatment plant receiving wastes from tannery industries in Italy. The strain was tentatively identified as a species of *Candida* based on morphological and physiological analyses. This strain was highly resistant to Cr(VI) when compared with eight other yeast species, growing at Cr(VI) concentrations of up to 500 μg/ml (10 mM). This resistance was constitutive. The Cr(VI)-resistant yeast did not reduce Cr(VI) to Cr(III) species under aerobic conditions. The yeast showed very little accumulation of Cr(VI). Consequently, the mechanism of resistance of the yeast to Cr(VI) appears to involve reduced accumulation of Cr, as has been shown in Cr(VI)-resistant bacteria.

Chromium compounds have widespread industrial uses in steel production, wood preservation, leather tanning, metal corrosion inhibition, paints and pigments, metal plating, and other applications (16). Consequently, Cr is a contaminant in certain waters, soils, and sediments, occurring primarily in the trivalent or hexavalent form (20). Although Cr is considered an essential trace element in animals and humans, elevated levels of Cr are toxic, and Cr(VI) compounds are mutagenic and carcinogenic in animals (7) and mutagenic in bacteria (17, 26). Cr(VI) is more toxic than Cr(III) to bacteria (17) and fungi (3). Therefore, Cr(VI) reduction might represent a detoxification process in microorganisms. A Cr(VI)-resistant strain of *Enterobacter cloacae* reduced Cr(VI) to Cr(III) under anaerobic growth conditions, which was attributed to use of chromate as an electron acceptor by the organism (27). Cells grown aerobically were also Cr(VI) resistant but did not reduce Cr(VI). Species of *Aeromonas* (10) and *Pseudomonas* (5, 8, 11) also reduce Cr(VI) to Cr(III), as do rat liver mitochondria (1). In *Pseudomonas fluorescens*, reduction of Cr(VI) appears to be unrelated to resistance (5). In addition, Cr is reduced by the metabolic products, such as hydrogen sulfide, of certain bacteria (22). Ascorbic acid and thiols (e.g., glutathione, cysteine, coenzyme M) also reduce Cr(VI) to Cr(III) at significant rates under physiological conditions (7).

Cr-resistant bacteria have been isolated from Cr-contaminated sediments (4, 10, 12), and plasmid-coded resistance to Cr(VI) has been reported in *Pseudomonas aeruginosa* (23), *P. fluorescens* (4), and *Alcaligenes eutrophus* (14). The mechanism of Cr resistance in the latter two strains involves reduced Cr(VI) uptake (14, 15). Chromate ion is accumulated intracellularly by the sulfate transport system (15). Horitsu et al. (8) found that a Cr(VI)-sensitive mutant strain of *Pseudomonas ambigua* accumulated six times more Cr than did the Cr(VI)-resistant parent strain. Cell extracts of the mutant also showed one-fourth of the Cr(VI)-reducing activity of the parent strain. Others who studied Cr accumulation by Cr(VI)-resistant bacteria did not make a connection between accumulation and resistance to Cr (6).

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The purpose of this study was to identify taxonomically a Cr(VI)-resistant yeast isolated from a Cr-contaminated environment and to study its mechanism of resistance to high Cr concentrations. Resistance to Cr has not been previously reported in yeasts.

MATERIALS AND METHODS

Sampling. Water samples were collected from the sewage treatment plant (Societa Gestione Scarichi) at Santa Croce sull'Arno in Tuscany, Italy. The plant received a mixture of domestic sewage and Cr-containing tannery wastes containing approximately 100 μg of Cr per ml. Water samples from the influent to the plant, from the activated sludge tank, and from the plant effluent to the Arno River were aseptically collected in whirl-pak (Nasco) bags and transported to the laboratory on ice.

Organism isolation and culture. Decimal dilutions (in sterile tap water) of sewage plant samples were added to duplicate test tubes containing sterile, molten glucose-peptone agar (pH 7.0), held at 45°C, consisting of (per liter of distilled water): 10 g of glucose, 5 g of polypeptone (Difco Laboratories, Detroit, Mich.), and 15 g of Bacto-Agar (Difco). The medium in the tubes was spiked with various amounts of an aqueous solution of $K_2Cr_2O_7$ (at neutral pH, dichromate ions are converted to chromate ions [20]) that had been filter sterilized (0.2-μm-pore-size filter) and added to the sterile molten agar to prevent problems associated with autoclaving chromate-containing solutions (2). The tubes were mixed briefly and immediately poured into petri dishes. Colonies were counted after 2 and 7 days of incubation at 28°C. It is possible that some die-off of organisms occurred (in molten agar) with the pour-plate method. Consequently, the numbers of Cr(VI)-resistant bacteria reported should be viewed on a relative or comparative basis.

An enrichment culture of Cr(VI)-resistant microorganisms was obtained by adding 1.0 ml of activated sludge to a conical flask containing 100 ml of glucose-peptone broth (pH 7.0) amended with 500 μg of Cr per ml (10 mM) as $K_2Cr_2O_7$. A Cr-resistant yeast isolate, designated strain DBVPG 6502, was studied morphologically and physiologically by the methods of van der Walt and Yarrow (24). All assays were carried out in liquid culture at 25°C, including assimilation studies, which were done with agitation on a roller drum.

TABLE 1. Yeast strains

Designation[a]	Species	Origin
DBVPG 6502	*Candida* sp.	Tannery waste
DBVPG 6016	*Candida famata*	Air
DBVPG 6406	*Candida famata*	Cheese
DBVPG 6140	*Candida guilliermondii*	Unknown
DBVPG 6050	*Debaryomyces hansenii*	Unknown
DBVPG 6404	*Debaryomyces hansenii*	Infected fingernail
DBVPG 6570	*Pichia ohmerii*	Unknown
DBVPG 6572	*Pichia guilliermondii*	Unknown
Vibar S.p.A.	*Saccharomyces cerevisiae*	Pharmaceutical

[a] DBVPG, Collection of industrial yeasts, Dipartimento di Biologia Vegetale, University of Perugia.

Fermentation of various sugars was detected by the appearance of gas in Durham tubes. Sporulation trials were done for various time periods of up to 3 months and at various temperatures ranging from 20 to 25°C on malt agar, yeast extract-glucose agar, corn meal agar, V8 agar (18), and acetate agar (13). Yeast morphology agar (Difco) was used for morphological tests. For comparison, a strain of *Saccharomyces cerevisiae* (commercial bakers' yeast; Vibar Nord S.p.A., Colico, Italy) and seven previously identified strains possessing phenotypic characteristics similar to strain DBVPG 6502 were studied (Table 1).

Scanning electron microscopy. Cells were fixed in glutaraldehyde (2% final concentration), filtered onto polycarbonate filters (pore size, 0.2 μm; Nuclepore Corp., Pleasanton, Calif.), dehydrated in a series of ethanol-water solutions (25, 50, 75, 80, 90, 95, and 100% ethanol, 2 min each), and critical point dried under a CO_2 atmosphere. The dried filters were mounted on scanning electron microscopy stubs and coated with gold (approximately 15.0 nm). The cells were examined (model 1000 scanning electron microscope; Amray, Bedford, Mass.) at an accelerating voltage of 20 kV.

nDNA extraction and analysis. The extraction and purification of nuclear DNA (nDNA) was carried out by the method of Price et al. (19) as modified by Vaughan Martini and Martini (25). The optical determination of nDNA-nDNA reassociation was determined by the method of Kurtzman et al. (9) on a model 250 spectrophotometer equipped with a model 2526 thermoprogrammer (Ciba-Corning Gilford Systems, Oberlin, Ohio).

Cr resistance studies. The MIC of Cr for strain DVBPG 6502 was determined by diluting 1.0 ml of actively growing culture into 99 ml of glucose-peptone broth. The diluted cultures of all strains were mixed, and 10-ml subsamples were added to 20-ml test tubes containing Cr(VI) (as $K_2Cr_2O_7$) or Cr(III) [$Cr_2(SO_4)_3$]. The tubes were incubated on a rotary drum at 28°C; after 22 h the A_{600} of the cultures was measured (UV-160 spectrophotometer; Shimadzu, Kyoto, Japan).

Cultures were tested for inducibility of Cr resistance by growth for eight successive transfers in Cr-free (as determined by atomic absorption spectrophotometry) glucose-peptone broth and comparison with strains growing in the presence of 100 μg of Cr per ml (1.9 mM) (as $K_2Cr_2O_7$) (induced cells). In these experiments, actively growing 12-h cultures of induced and uninduced cells were diluted 1:10 into 250-ml Klett flasks containing 45 ml of glucose peptone broth amended with 100 μg of Cr per ml (as $K_2Cr_2O_7$), and absorbance increases were monitored with time by using a Klett-Summerson colorimeter (no. 66 filter).

Cr reduction assays. Cells were grown in glucose-peptone broth to the midlogarithmic phase, centrifuged (4,000 × *g* for 15 min), and washed twice in 10 mM PIPES [piperazine-*N*,*N*-bis(2-ethanesulfonic acid)] buffer (pH 7.2) and suspended in PIPES buffer containing 1% (wt/vol) glucose and 10 μg of Cr per ml (as $K_2Cr_2O_7$). Two methods were used to measure Cr(VI) in culture solutions (1). Briefly, method 1 involved combination of 0.75 ml of culture supernatant with 0.75 ml of a Cr(VI) extractant solution. The extractant solution contained Amberlite liquid anion-exchange resin (LA-2; Sigma Chemical Co., St. Louis, Mo.) (100 ml), 1:1 HCl-deionized water (50 ml), and methylisobutylketone (to bring the volume to 250 ml). The solution was stirred continuously to maintain homogeneity. The combined culture supernatant-extractant solution was shaken for 2 min on a Vortex mixer and centrifuged for 3 min at 12,000 × *g*, and the supernatant was analyzed for Cr by graphite furnace atomic absorption spectrophotometry (model 460 spectrophotometer; The Perkin-Elmer Corp., Norwalk, Conn.) employing graphite furnace (Perkin-Elmer model HGA 2100) atomization and a Cr hollow-cathode lamp. Method 2 involved extraction of 0.25 ml of culture supernatant with 0.8 ml of diphenylcarbazide solution and measurement of the A_{540} of the solution. A variation of this method involved extraction of the solution with Amberlite followed by the addition of diphenylcarbazide solution and measurement of A_{550}. In addition, the absorbance spectra of the culture supernatants were measured directly by UV-visible spectrophotometry (model 330 spectrophotometer; Perkin-Elmer). Cr(VI) species in aqueous solution absorb at 340 to 360 nm, whereas Cr(III) species do not.

Cr accumulation studies. Actively growing cultures of strain DBVPG 6502 and *S. cerevisiae* in glucose-peptone broth (37°C, agitation at 300 rpm) were washed twice (centrifugation at 3,600 × *g* for 15 min) in 10 mM PIPES buffer (pH 7.2) and suspended in PIPES. The cell suspension was added to a 250-ml conical flask containing 20 ml of PIPES buffer amended with 10 μg of Cr per ml (as $K_2Cr_2O_7$) and 1% (wt/vol) glucose to give a cell concentration of about 1.0 mg (dry weight) per ml. A duplicate flask contained 1.0 mM sodium azide (NaN_3), and a control flask contained cells without Cr.

The cells were incubated at 37°C with shaking at 300 rpm; 0.5-ml samples were removed at various times and washed twice in Cr-free PIPES (centrifugation at 12,000 × *g* for 3 min). The washed pellet was digested for 60 min at 90°C in 0.5 ml of HNO_3-water (1:1), and the resulting solution was analyzed for Cr by graphite furnace atomic absorption spectrophotometry.

RESULTS

Samples of wastewater collected from three different areas of the sewage treatment plant contained Cr-resistant microorganisms. The highest percentage of Cr(VI)-resistant microorganisms was found in the influent, although the largest number of resistant microorganisms occurred in samples of the activated sludge (Fig. 1). Only a fraction of a percent of the microbial population of these samples grew on a glucose-peptone agar containing 100 μg of Cr(VI) per ml. Lull et al. (12) also found that <0.1% of the viable plate count from Cr-contaminated river sediments grew on agar medium containing 100 μg of Cr per ml.

A Cr(VI)-resistant yeast was isolated from an enrichment culture inoculated with sludge and containing 500 μg of Cr per ml (Fig. 2). The results of morphological and physiological analyses have shown significant similarities as well as differences between the Cr-resistant isolate, strain DBVPG

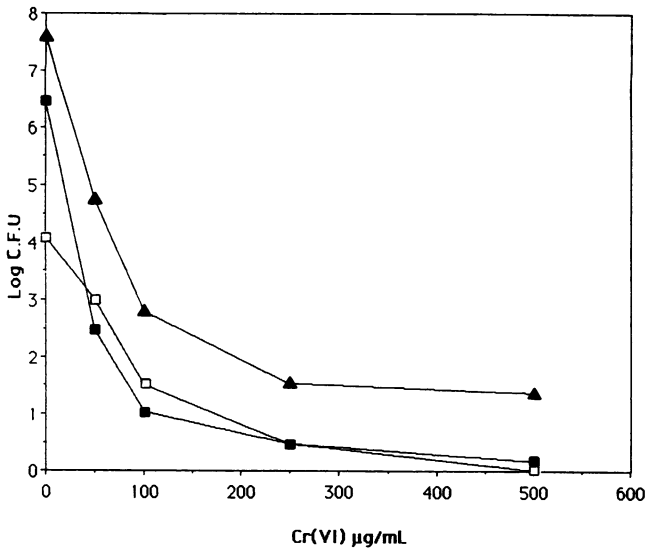

FIG. 1. Numbers of cells (log CFU) from sewage treatment plant influent (□), activated sludge (▲), and effluent (■) growing on glucose-peptone agar amended with various concentrations of Cr(VI).

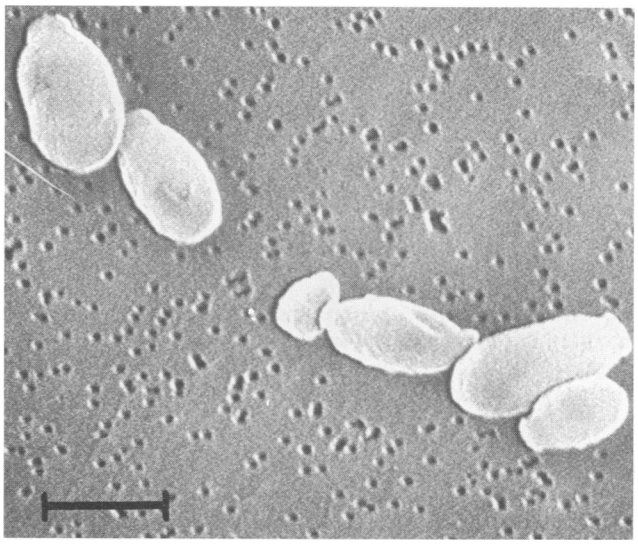

FIG. 2. Scanning electron micrograph of *Candida* sp. strain DVBPG 6502. Bar, 2.0 µm.

6502, and other known yeast species considered in this study. Extensive morphological and physiological tests were conducted comparing DBVPG 6502 with other yeasts, including assimilation and fermentation tests on 37 sugars, organic acids, and other compounds. Only the most significant taxonomic properties are reported in Table 2. A close phenotypic affinity of DBVPG 6502 with the species *Pichia ohmeri* and *P. guilliermondi* was noted (Table 2). However, significant differences in pseudomycelium formation and cell size probably exclude the unknown isolate from these species. A certain affinity could also be seen with the species *Debaryomyces hansenii* and *Candida famata*. Also in these cases, differences in the assimilation of lactose, melizitose, and rhamnose seem to indicate divergence.

Limited affinity of DBVPG 6502 with the above species was confirmed further with optical nDNA-nDNA reassociations. No significant homology was found between DBVPG 6502 and any of the strains tested (Table 3), indicating significant evolutionary divergence.

Strain DBVPG 6502 was resistant to Cr(VI) in liquid culture, with growth inhibited by 50% at concentration of about 300 µg of Cr per ml. The organism exhibited a similar level of resistance to Cr(III) (Fig. 3). Only about 0.1% of the microorganisms in the Cr-contaminated activated sludge sample were resistant to 50 µg of Cr (VI) per ml on glucose-peptone agar. In comparison, *S. cerevisiae* was almost completely inhibited at a concentration of 5 µg of Cr(VI) per ml; Cr(III) was much less toxic (Fig. 3b), as has been shown with other organisms (3, 17). Furthermore, seven additional yeast strains, with morphological and physiological characteristics similar to those of strain DBVPG 6502, were much more sensitive than strain DBVPG 6502 to Cr(VI) (Fig. 4).

The resistance of DBVPG 6502 to Cr(VI) was constitutive. Cells cultured in the absence of Cr for eight transfers and then reinoculated into Cr-containing media showed no difference in the lag phase when compared with that of a culture carried on 100 µg of Cr(VI) per ml (Fig. 5).

There was no significant reduction of Cr(VI) to Cr(III) by strain DBVPG 6502. No decrease in Cr(VI) concentration was detected upon analysis of culture liquids for Cr(VI) by graphite furnace atomic absorption spectrophotometry after extraction of the Cr(VI) with an Amberlite liquid anion exchanger and by the A_{540} of the DPC-Cr(VI) complex. Neither did direct absorbance spectra taken in the region of 340 to 360 nm reveal a loss of Cr(VI) from solution.

Cells of DBVPG 6502 accumulated very little Cr, whereas *S. cerevisisae* exhibited much higher Cr accumulation (Fig.

TABLE 2. Salient properties of strains studied

DBVPG strain[a]	Glucose fermentation	Assimilation[b] of:							Growth at 37°C	Vitamin free[c]	Cr(VI) resistance	Cell size (µm)	Sporulation	
		Lactose	Melibiose	Melezitose	Rhammose	Citric acid	D-Gluconate	Glucosamine					No. of spores	Characteristics
6502	+	−	+	−	−	+	+	+	+	+	+	1–3 by 1–4	0	
6016	+	+	+s	+s	−	v	−	+w	−	−	−	2–5 by 4–8	0	
6050	+	+	+s	+	+s	+	−	−	−	−	−	2–6 by 2–8	1–2	Warty
6570	+	−	−	−	−	+	ND	+	+	−	−	2–6 by 2–25	1–4	Hat shaped
6572	+	−	+	+	v	v	+	+	+	−	−	1–5 by 2–15	1–4	Hat shaped

[a] Strain numbers refer to the collection of the Dipartimento di Biologia Vegetale, Universita di Perugia, Italy: 6502, unknown *Candida* species; 6016, type strain of *C. famata*; 6050, type strain of *D. hansenii*; 6570, *P. ohmeri*; 6572, *P. guilliermondii*.

[b] +, Growth in 7 days at 25°C; −, no growth; +s, growth after 14 days; +w, weak growth after 21 days; v, variable; ND, not determined.

[c] Growth in yeast nitrogen basal medium without vitamins.

TABLE 3. nDNA-nDNA reassociation between yeast strains

Species and strain	% nDNA-nDNA homology with DBVPG strain			
	6502	6050	6404	6016
D. hansenii DBVPG 6050	25			
D. hansenii DBVPG 6404	23	21		
C. famata DBVPG 6016	33	30	4	
C. famata DBVPG 6406	16	50	67	16

6). The presence of sodium azide (1.0 mM) in the growth medium did not affect the rate or extent of Cr accumulation with DBVPG 6502. The early Cr(VI) uptake kinetics were the same for azide-treated and untreated *S. cerevisiae*. However, after 40 min, uptake of Cr(VI) was more rapid in cells not exposed to azide.

DISCUSSION

Bacteria resistant to Cr(VI) have been isolated from Cr-containing environments (11, 12). Plasmid-mediated resistance to Cr(VI) in *P. fluorescens* and *Alcaligenes eutrophus* involves reduced accumulation of the metal (14, 15). Cr(VI) reduction by bacteria to less toxic Cr(III) has been reported (5, 10, 11, 27), but a connection between reduction and Cr resistance has not been established.

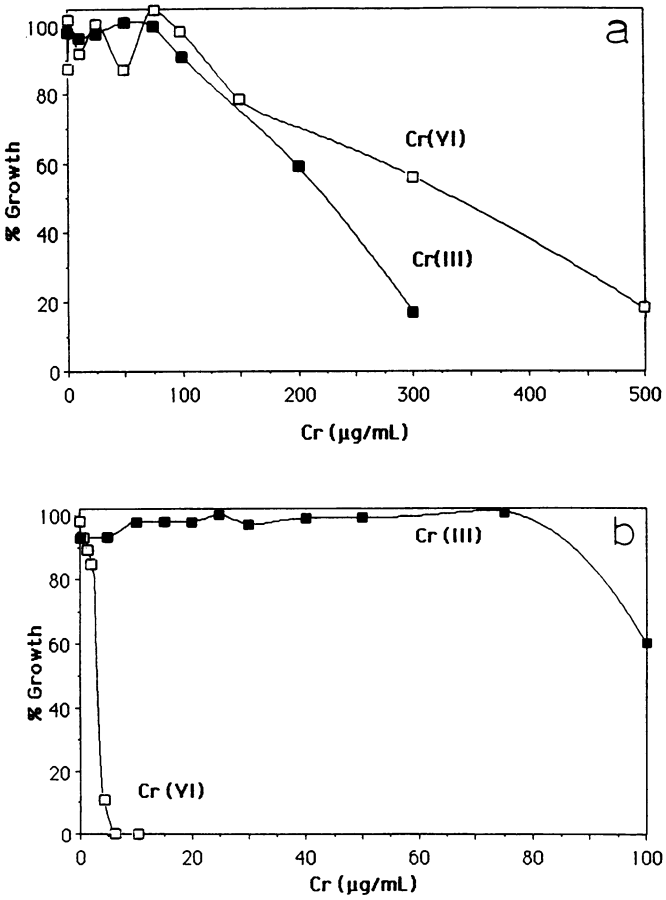

FIG. 3. MIC of Cr(VI) (□) and Cr(III) (■) for *Candida* sp. strain DBVPG 6502 (a) and *S. cerevisiae* (b). Actively growing cultures were diluted 1:100 into fresh medium containing Cr(VI) or Cr(III). After incubation for 22 h, the A_{600} of the culture was measured.

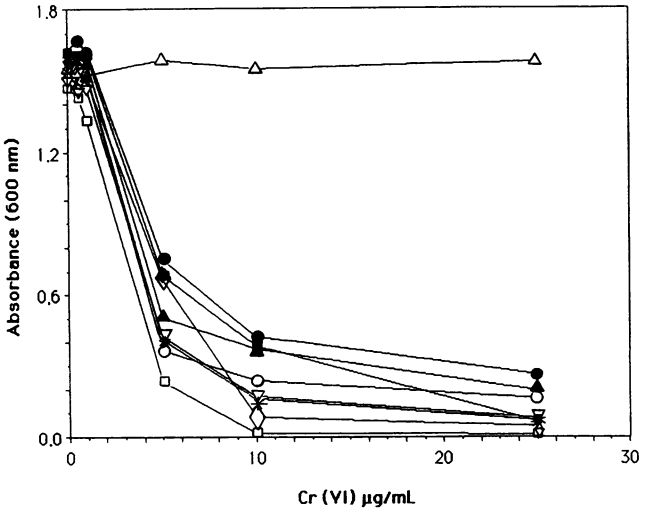

FIG. 4. MIC of Cr(VI) for the following DBVPG strains: △, 6502; ●, 6570; ▲, 6404; ○, 6050; ■, 6572; *, 6016; ◇, 6406; □, 6140. ▽, *S. cerevisiae*. See Table 1 for strain designations.

Strain DBVPG 6502 is highly resistant to Cr(VI); it can grow in the presence of 300 µg of Cr(VI) per ml. Only a small fraction of microorganisms in the Cr-contaminated wastewater were resistant to this level of Cr. Yeasts in general are not resistant to Cr(VI), as shown by results with eight different species (Fig. 3 and 4).

No reduction of Cr(VI) by strain DBVPG 6502 was detected, indicating that reduction is not the mechanism of Cr(VI) resistance in the organism. Rather, the organism accumulated very little Cr from solution, in contrast to a culture of *S. cerevisiae*, which was sensitive to Cr(VI) and accumulated substantial Cr(VI). Thus, the mechanism of resistance to Cr(VI) in strain DBVPG 6502 appears to involve lowered accumulation of Cr as reported for the bacteria *P. fluorescens* and *A. eutrophus* (14, 15).

Metal accumulation in microorganisms is characterized by

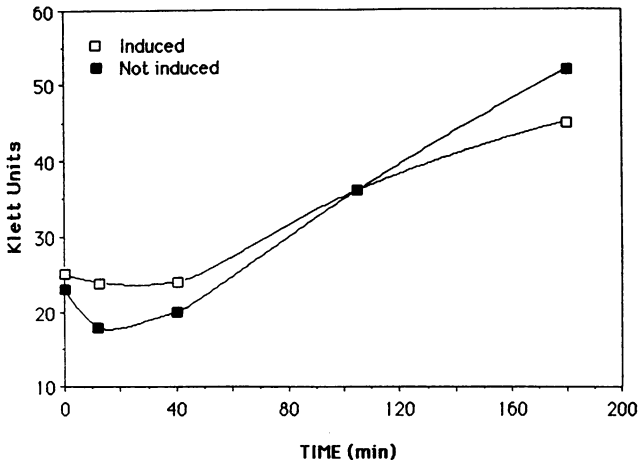

FIG. 5. Growth of induced (□) and uninduced (■) *Candida* sp. strain DBVPG 6502 in the presence of 100 µg of Cr(VI) per ml. Actively growing cells carried for eight transfers in Cr-free glucose-peptone broth (uninduced) or grown in broth containing 100 µg of Cr(VI) per ml (induced) were diluted 1:10 into broth containing 100 µg of Cr(VI) per ml.

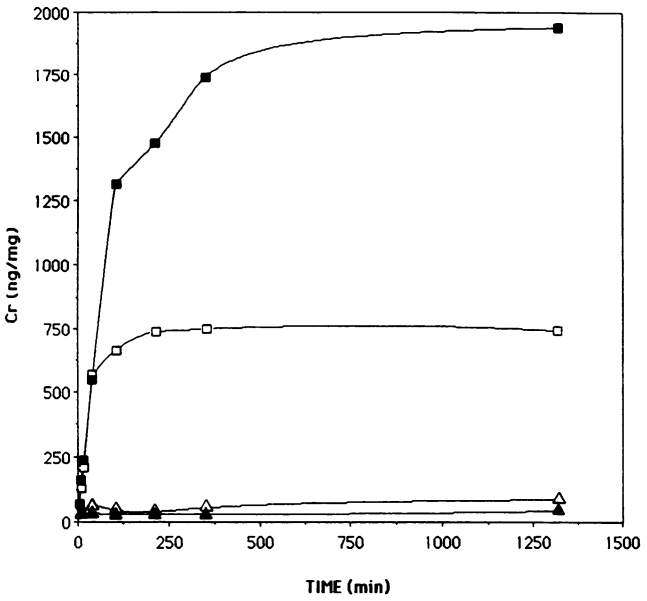

FIG. 6. Cr(VI) accumulation by *Candida* sp. strain DBVPG 6502 (△, ▲) and *S. cerevisiae* (□, ■). Actively growing cells were washed and suspended in flasks containing 10 mM PIPES buffer, 10 μg Cr(VI)/ml, and in some cases 1.0 mM NaN_3 (△, □). Cells were periodically removed, digested in acid, and analyzed for Cr content by graphite furnace atomic absorption spectrophotometry.

a two-phase process involving first a rapid, reversible, metabolism-independent binding, followed by energy-dependent accumulation by specific carrier systems. Sometimes toxic ions bear close chemical similarities to nutrient ions and are mistakenly accumulated by cells, as occurs with arsenate-phosphate and Cd^{2+}-Mn^{2+} systems (21). Chromate is accumulated by the sulfate transport system (15). Energy-dependent Cr(VI) uptake was indicated in Cr(VI)-sensitive *S. cerevisiae*. Initially, azide-treated and untreated cells rapidly accumulated Cr(VI); however, untreated cells accumulated more Cr(VI) after the initial rapid binding phase. In contrast, DBVPG 6502 accumulated very little Cr in the presence or absence of azide. This suggests that the reduced accumulation does not involve metabolism-dependent efflux (21). Rather, the results suggest that the cell envelope of DBVPG 6502 could be different from that of *S. cerevisiae* in its chemical affinity for Cr(VI) and/or that metabolism-dependent transport of Cr(VI) does not occur in strain DBVPG 6502.

The Cr-resistant isolate designated as strain DBVPG 6502, although showing some phenotypic affinity to some known species of the genera *Pichia*, *Debaryomyces*, and *Candida*, demonstrated differences significant enough to consider it a separate species. In addition, an impermeability of the isolate to chromate, a property not reported in yeast strains, may also support consideration of the isolate as a new species. Since sporulation studies (ongoing) have not revealed a sexual cycle of this strain, it will be considered a new species of the genus *Candida* until such time as sporulation can be verified.

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