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Hexavalent-chromium reduction by a chromate-resistant *Bacillus* sp. strain

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Abstract

Bacillus strain QC1-2, isolated from a chromium-polluted zone, was selected by its high ability to both tolerate and reduce hexavalent chromium [Cr(VI)] to less-toxic trivalent chromium [Cr(III)]. Cell suspensions of strain QC1-2 rapidly reduced Cr(VI), in both aerobic and anaerobic conditions, to Cr(III) which remained in the supernatant. Cr(VI) reduction was dependent on the addition of glucose but sulfate, an inhibitor of chromate transport, had no effect. Studies with permeabilized cells and cell extracts showed that the Cr(VI) reductase of strain QC1-2 is a soluble NADH-dependent enzyme.

Introduction

The presence of chromate [CrO_4^{2-}; hexavalent chromium, Cr(VI)] in the environment inhibits most bacteria and also selects the appearance of resistant variants (Cervantes 1991). Chromosome-encoded resistance to chromate results from alterations in sulfate transport, since chromate is taken up mainly by this pathway (Ohtake et al., 1987). Bacterial chromate resistance is commonly conferred by plasmids (Ohtake et al. 1987; Bopp & Ehrlich 1988; Cervantes & Ohtake 1988; Cervantes & Silver 1992).

Reduction of Cr(VI) to the less-toxic trivalent chromium [Cr(III)] form can be considered as an additional chromosome-determined mechanism of resistance to chromate. Cr(VI) reduction has been reported in several bacterial species (Gvozdyak et al. 1986; Horitsu et al. 1987; Bopp & Ehrlich 1988; Wang et al. 1989; Das & Chandra 1990; Ishibashi et al. 1990). Chromate reduction by bacteria occurs under aerobic or anaerobic conditions and it has been associated with soluble or membrane-bound enzyme activities (Bopp & Ehrlich 1988; Wang et al. 1990; Ishibashi et al. 1990; Suzuki et al. 1992). Bacterial chromate reduction has the potential to become a useful bioremediation system. In this paper, we report the partial characterization of the chromate reduction process from a *Bacillus* strain isolated from a chromium-polluted area.

Materials and methods

Bacterial strains

Chromate-resistant bacteria were isolated from soil and discharge water samples obtained from the surroundings of a large chromium-processing factory near the City of Leon, Gto. in Central México. Twenty-six isolates, able to grow in the presence of up to 750 μg/ml potassium chromate (2.3 mM CrO_4^{2-}) in Mueller-Hinton agar (Bioxon de México) after a 24-h incubation at 30° C, were considered as chromate-resistant.

Other bacteria used were chromate-resistant *Pseudomonas aeruginosa* PU21 (pUM505) (Cervantes & Ohtake 1988) and chromate-sensitive *Bacillus subtilis* 1A-46 (pSK263) (a gift of G. Ji, University of Illinois, Chicago).

Strain identification

Strain QC1-2 was assigned to the genus *Bacillus* by the results of the following tests: Gram-positive staining of long-chain grouped bacilli, formation of spores able to withstand heating at 70° C for 10 min, a positive catalase test, motile aerobic and facultative anaerobic cells (Holt 1977).

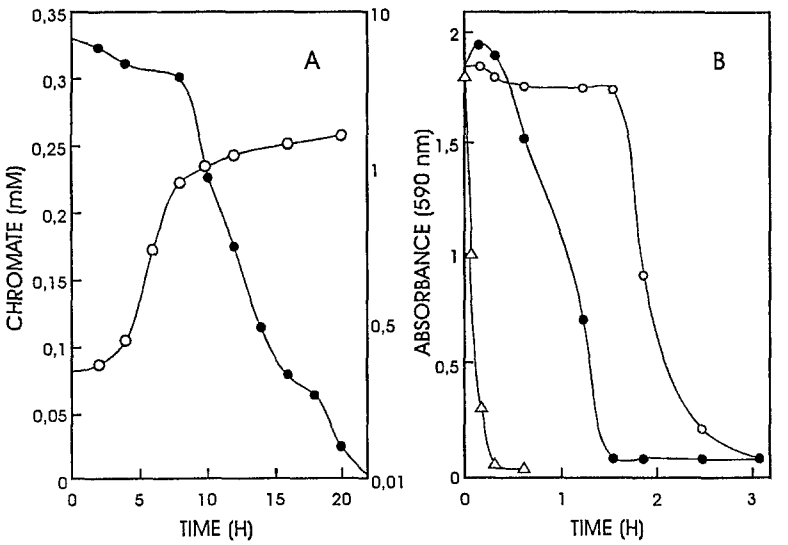

Fig. 1. Chromate reduction and resistance by *Bacillus* QC1-2. A. Cr(VI) (•) was measured at intervals during growth (○) of strain QC1-2 in Luria broth at 30° C with shaking. B. Chromate susceptibility of strains *Bacillus* QC1-2 (○); *Pseudomonas aeruginosa* PU21 (pUM505) (•); and *Bacillus subtilis* 1A-46 (pSK263) (△). Cultures were grown for 18 h as above with the indicated concentrations of potassium chromate and the turbidity was determined.

Susceptibility to chromate

Strains were inoculated in Luria broth (Sigma Chemical Co.) in the presence of varying concentrations of chromate. Cultures were incubated for 18 h at 30° C with shaking and the turbidity at 590 nm was measured.

Determination of chromate reduction

For measurement of chromate reduction, bacteria were grown in Luria broth containing adequate amounts of chromate. After incubation at 30° C with shaking, cultures were centrifuged and the remaining Cr(VI) was determined in the supernatant fluid. Hexavalent chromium and total chromium [Cr)VI) plus Cr(III)] were quantified with diphenylcarbazide (Merck) and a spectrophotometric method (American Public Health Association 1971).

For cell suspensions, bacterial pellets were washed twice with 100 mM sodium phosphate buffer (pH 7.0) containing 0.81 mM $MgSO_4$ and 0.5% glucose (Ishibashi et al. 1990) and suspended at an absorbance (590 nm) of 1.8 in the same buffer. The buffer lacked either sulfate or glucose when testing its effect on Cr(VI) reduction. After adding 0.3 mM Cr(VI), suspensions were incubated and treated as above. Cr(VI) reduction in anaerobiosis, was similarly tested except that tubes were completely filled with the cell suspensions and the incubation was without shaking.

Permeabilization of cells

A modification of the procedure of Basabe et al. (1979) was used. Overnight cultures of *Bacillus* QC1-2 in Luria broth were harvested by centrifugation, washed twice with 100 mM Tris-HCl (pH 7.5), suspended in a tenth-volume of the buffer and were mixed with either (final concentration) 1% toluene, 2% Triton X-100, 1% sodium dodecyl sulfate or 1% Tween-80. Mixtures were vortexed for six-20 sec intervals, washed three times with buffer and suspended to an absorbance (590 nm) of 1.8. After adding Cr(VI) and NADH (1 mM), the permeabilized cells were incubated at 30° C for the determination of chromate reducing activity.

Cell extracts

Bacterial pellets, prepared by centrifugation of 500-ml cultures of *Bacillus* QC1-2, were washed twice in 100 mM Tris-HCl (pH 7.0) and disrupted by freezing with liquid nitrogen and grinding in a mortar. Cell extracts were diluted with the same buffer and frac-

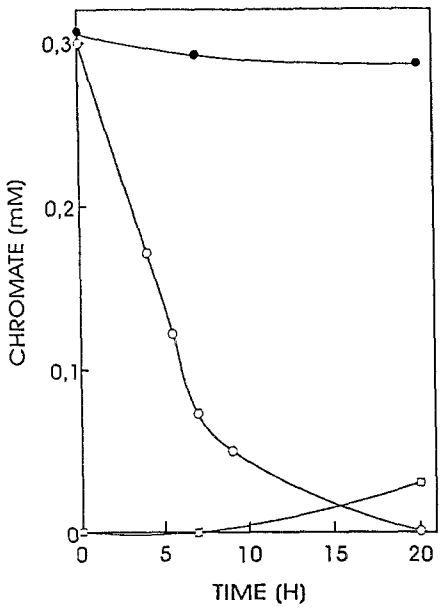

Fig. 2. Chromate reduction by *Bacillus* QC1-2 suspensions. Cell suspensions (ca. 1×10^9 cells/ml) containing 0.3 mM CrO_4^{2-} were incubated at 30° C for indicated times. After centrifugation, Cr(VI) was quantified in the supernatant (o) and in the pellet (□); total chromium [Cr(VI) + Cr(III)] was measured in the supernatant (•).

tionated by centrifugation at 100,000 × g for 1 h at 4° C. Both the supernatant fluid and the remaining pellet were further tested for Cr(VI) reduction activity. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Results and discussion

We isolated 26 chromate-resistant bacterial strains from a chromium-polluted zone. All of the strains were able to reduce Cr(VI) to Cr(III), although at different rates (data not shown). A *Bacillus* strain, called QC1-2, was selected by its higher ability to both reduce and tolerate chromate. Aerobic incubation of cultures of QC1-2 with chromate caused the complete disappearance of 0.33 mM CrO_4^{2-} in 22 h (Fig. 1A). A similar rate of Cr(VI) reduction was obtained under anaerobic conditions (data not shown).

The finding that chromate-resistant as well as chromate-sensitive bacterial isolates are able to reduce Cr(VI) may be related to the involvement of chromate reductases in normal metabolism (Ishibashi et al. 1990). Horitsu et al. (1987) reported the isolation of chromate-sensitive mutants from a rapid Cr(VI)-reducer *Pseudomonas ambigua* strain, but they still retained a low chromate-reducing activity.

Table 1. Chromate reductase activity from *Bacillus* QC1-2.

System[a]	Treatment	Specific activity[b]
Intact cells	None	4.30
Permeabilized cells	1% Toluene	21.80
	2% Triton X-100	26.10
	1% SDS	13.00
	1% Tween-80	13.00

[a] Prepared as described in Methods; assays contained 0.15 mM CrO_4^{2-} and 1 mM NADH.
[b] nmol CrO_4^{2-}/min per mg protein. Values represent the mean of two experiments.

Chromate reductases from several bacterial species have been reported, although only a few have been characterized; these include those from species of *Pseudomonas* (Bopp & Ehrlich 1988; Ishibashi et al. 1990; Suzuki et al. 1992) and *Enterobacter* (Wang et al. 1990).

As shown in Fig. 1B, *Bacillus* QC1-2 was highly resistant to chromate as compared to *Bacillus subtilis* 1A-46 (pSK263) as well as to *Pseudomonas aeruginosa* PU21 carrying chromate-resistance plasmid pUM505 (Cervantes & Ohtake 1988). Although strain QC1-2 was able to both reduce (Fig. 1A) and tolerate (Fig. 1B) chromate, these two properties may be unrelated. Bopp & Ehrlich (1988) found that plasmid-mediated resistance to chromate was independent of chromate reduction in *Pseudomonas fluorescens* LB300. Moreover, chromate-sensitive *P. putida* PRS-2000 reduced chromate efficiently (Ishibashi et al. 1990). On the other hand, chromate-resistant *Enterobacter cloacae* HO1 reduced chromate under anaerobic conditions but not in the presence of oxygen (Wang et al. 1989).

A faster elimination of Cr(VI) was achieved using washed suspensions of strain QC1-2 (Fig. 2), which also shows that disappearance of chromate from the supernatant was caused by its transformation to Cr(III), since total chromium [Cr(VI) plus Cr(III)] was unchanged throughout the assay. No significant amount of chromium was detected in the bacterial pellet (Fig. 2), thus chromate is not trapped by the cells.

Chromate reduction by cell suspensions of QC1-2 was dependent on the presence of glucose (Fig. 3A), but sulfate, a competitive inhibitor of chromate trans-

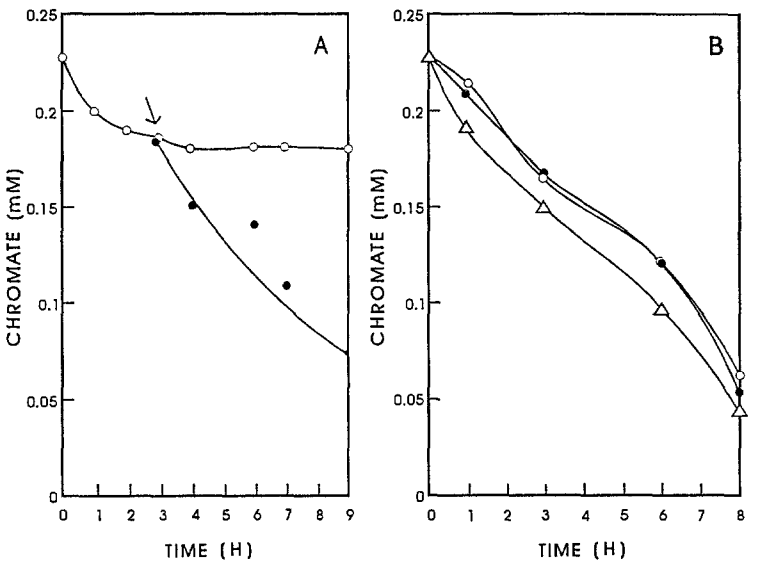

Fig. 3. Effect of glucose and sulfate on chromate reduction by *Bacillus* QC1-2. A. Cell suspension without glucose (o) or with 0.25% glucose added (arrow) after a 3-h incubation (•). B. Cell suspensions without sulfate (o) or with 1 mM (•) or 10 mM (△) magnesium sulfate. Remaining Cr(VI) was measured as in Fig. 2.

port (Ohtake et al. 1987), had no effect on the rate of reduction even at a 40-fold higher concentration (Fig. 3B). Similar results were obtained by Ishibashi et al. (1990) in *P. putida*. Lacking of an effect of sulfate on chromate reduction suggests that chromate transport is not involved and that Cr(VI) reduction probably takes place in the cell envelope and not in the cytosol, unless a novel chromate transport pathway is functioning. Protoplasts of *Bacillus* QC1-2 efficiently reduced Cr(VI) (data not shown), ruling out the presence of the chromate reductase in the wall-membrane space. Chromate reduction was significantly stimulated in permeabilized cells of *Bacillus* QC1-2 (Table 1), suggesting that the chromate reductase is a soluble enzyme not tightly bound to the cell membrane.

Fractionation of cell extracts of *Bacillus* QC1-2 resulted in the recovery of a chromate-reducing activity from the supernatant fluid, whereas a low activity was detected in the membrane fraction (Fig. 4). These data confirm the soluble nature of the reductase which resembles in this respect the enzymes from *P. ambigua* (Horitsu et al. 1987) and *P. putida* (Ishibashi et al. 1990).

The crude enzyme from the supernatant fluid was used to determine some catalytic properties of the chromate reductase. This activity showed a clear dependence on chromate (Fig. 5A). An apparent Michaelis-Menten constant (K_m) of 1.25 mM CrO_4^{2-} and a maximum velocity (V_{max}) of 416 nmol CrO_4^{2-}/min per mg of protein were obtained from Lineweaver-Burk plots (Fig. 5A, inset). NADH was needed as a substrate for electron transfer, showing a cooperative sigmoidal

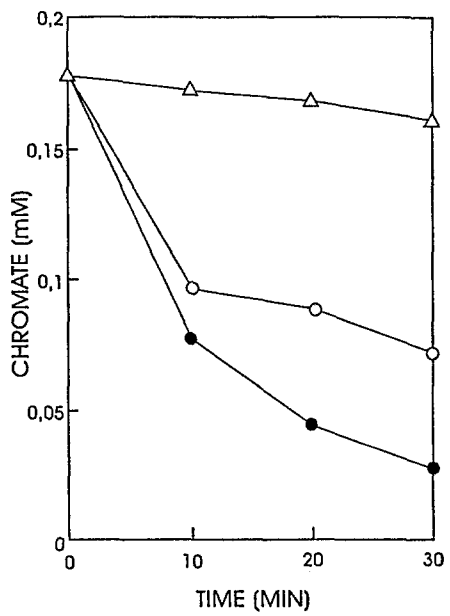

Fig. 4. Chromate reduction by cell extract fractions of *Bacillus* QC1-2. Cell extract was fractionated by centrifugation (100,000 × g/1 h) and the activity of chromate reductase was measured following incubation with 180 μM CrO_4^{2-} at 32° C. Whole extract (o), supernatant fluid (•), pellet (△).

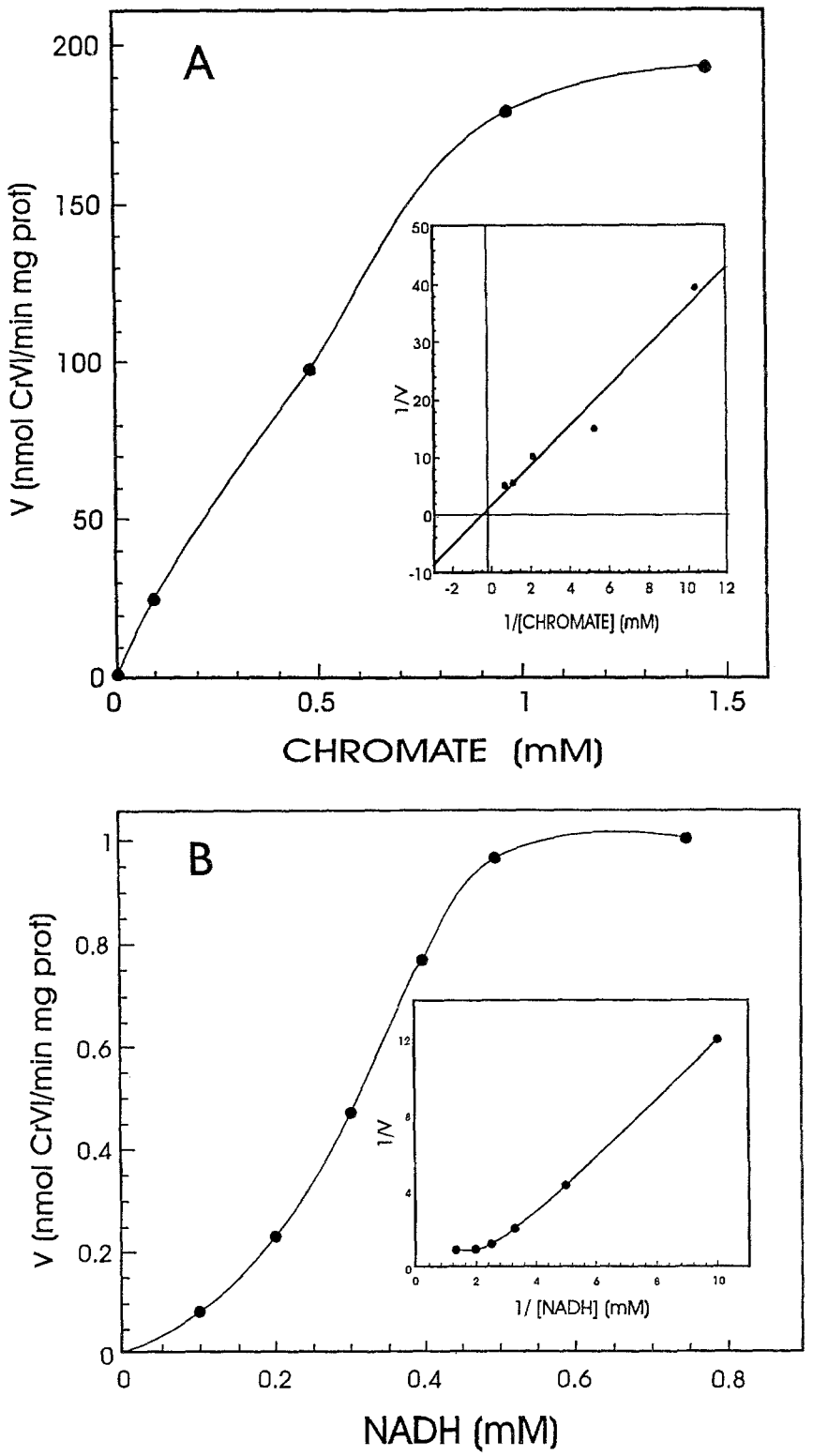

Fig. 5. Dependence on substrate and electron donor by the chromate reductase of *Bacillus* QC1-2. Crude enzyme preparations (500 μg protein) from the supernatant fluid of the cell extract were incubated with the indicated amounts of (A) CrO_4^{2-}, plus 100 μM NADH or (B) NADH, plus 250 μM CrO_4^{2-}, for 10 min at 32° C and chromate reduction was measured as described in Methods. Insets: double reciprocal plots.

curve (Fig. 5B). A double reciprocal plot rendered a biphasic curve (Fig. 5B, inset), suggesting an allosteric nature of Cr(VI) reductase.

Chromate-reductase activities from Gram-negative bacteria have been localized either in the cell soluble fraction (Horitsu et al. 1987; Ishibashi et al. 1990) or associated to the cell membrane (Bopp & Ehrlich 1988; Wang et al. 1989). Chromate reduction has also been observed in Gram-positive species (Gvozdyak et al. 1986; Das & Chandra 1990) but these reductases have not yet been characterized.

Recent interest in bacterial Cr(VI) reduction has been evoked by the potential use of this process with bioremediation purposes (Cervantes 1991; Cervantes & Silver 1992). Given the notable properties of *Bacillus* QC1-2 to both tolerate and reduce Cr(VI), we are currently analyzing the ability of this non-pathogenic soil bacterium to detoxify chromate from chromium-containing industrial discharges.

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