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Aerobic chromate reduction by *Bacillus subtilis*

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Summary

We have studied the reduction of hexavalent chromium (chromate) to the less toxic trivalent form by using cell suspensions and cell-free extracts from the common soil bacterium, *Bacillus subtilis. B. subtilis* was able to grow and reduce chromate at concentrations ranging from 0.1 to 1 mM K_2CrO_4. Chromate reduction was not affected by a 20-fold excess of nitrate-compound that serves as alternate electron acceptor and antagonizes chromate reduction by anaerobic bacteria. Metabolic poisons including sodium azide and sodium cyanide inhibited chromate reduction. Reduction was effected by a constitutive system associated with the soluble protein fraction and not with the membrane fraction. The reducing activity was heat labile and showed a K_m of 188 μm CrO_4^{2-}. The reductase can mediate the transfer of electrons from NAD(P)H to chromate. The results suggest that chromate is reduced via a detoxification system rather than dissimilatory electron transport.

Introduction

Chromium (Cr) is an essential micronutrient required for the growth of many organisms. However, at high concentrations, Cr is toxic, mutagenic, carcinogenic and teratogenic (Komori et al. 1990; Luli et al. 1983).

Of the two stable Cr oxidation states, Cr(VI), apart from being the predominant species involved in mutagenicity, carcinogenicity, and teratogenicity (Petrilli & De Flora 1977), is approximately 100 times more toxic than Cr(III), a form considered relatively innocuous (Luli et al. 1983). Moreover, while Cr(VI) is highly soluble in water and forms divalent oxyanions [chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$)], Cr(III) readily forms less-soluble chromium hydroxides at neutral pH (Bell & Lott 1966). The biological effects of chromates appear to be related to its uptake, since while Cr(VI) passes easily through cellular membranes, the trivalent form is unable to cross through biological membranes (Arslan et al. 1987).

Wastewaters containing chromates are generated in many industrial processes including manufacture of metallic alloys (the main use of Cr), chrome leather tanning, metal cleaning processing, wood preservation, ceramic, pyrotechnics, electronics and so on (Cervantes 1991; Ohtake et al. 1990a).

Conventional methods for removing chromates from effluents include chemical reduction followed by precipitation, ion exchange and adsorption on coal, activated carbon, alum, kaolinite and flyash (Ohtake et al. 1990a). Nevertheless, these methods are expensive due to their requirements for high energy or large quantities of chemical adsorbents.

Some chromate resistant bacteria have been shown to reduce chromate to the trivalent form, including strains of *Pseudomonas* (Bopp & Ehrlich 1988; Horitsu et al. 1987; Ishibashi et al. 1990; Suzuki et al. 1992), *Aeromonas* (Kvasnikov et al. 1985), *Enterobacter* (Ohtake et al. 1990a, b; Wang et al. 1989, 1990, 1991), and other species (Gvozdyak et al. 1986). Chromate reduction occurs both in anaerobic (chromate being used as final electron acceptor) and aerobic conditions. Anaerobic chromate reduction occurs with a membrane preparation (Komori et al. 1989; Wang et al. 1989, 1990). On the other hand, the aerobic chromate reductase activities found in other bacteria (Bopp & Ehrlich 1988; Horitsu et al. 1987) probably involve soluble proteins (Ishibashi et al. 1990). This bacterial

capacity to reduce Cr(VI) has potential not only for detoxification but also for the removal of chromates from water.

It has also been shown (Efstathion & McKay 1977; Novic & Roth 1968) that there are many organisms that although resistant to high concentrations of Cr(VI) do no alter the Cr redox state.

To our knowledge, apart from a brief mention to the capacity of some Gram-positive bacteria (including a strain of *Bacillus subtilis*) to reduce chromate under anaerobic conditions, all studies on chromate reduction have been carried out using Gram-negative bacteria (mostly, *Pseudomonas* and *Enterobacter* strains). Consequently, this is the first report on the factors affecting chromate reduction by a Gram-positive organism under aerobic conditions.

Methods

Microorganism and growth conditions

B. subtilis $168t^+$, a reverted prototroph of auxotroph thy^- obtained from Marburg strain (Spanish Type Culture Collection, CECT No. 461), was used throughout this study. The organism was cultured on a minimal chemically defined liquid medium that contained: 13.9 g l^{-1} K_2HPO_4, 6.0 g l^{-1} KH_2PO_4, 2.0 g l^{-1} $(NH_4)_2SO_4$, 1.9 g l^{-1} $C_6H_5Na_3O_7$. $2H_2O$ (tri-sodium citrate dihydrate), 23.6 mg l^{-1} $Ca(NO_3)_2$. $4H_2O$, 19.8 mg l^{-1} $MnCl_2$, 0.28 mg l^{-1} $FeSO_4$. $7H_2O$ and 1% (w/v) glucose. Cultures were grown in 250 ml Erlenmeyer flasks with continuous shaking (250 r.p.m.). Minimal medium plates were made by combining the above specified ingredients with 1.5% (w/v) agar (Difco). The growth temperature was 30 °C.

Growth was initiated in shaked flasks by using inocula from minimal medium plates. Before initiating liquid medium experiments, cultures were transferred twice into fresh medium, over a period of approximately 24 hours. Reinoculations were timed to ensure that cultures were under excess nutrient conditions, and thus in an environment allowing balanced exponential growth. By using this pre-inoculation procedure, no lag phase was observed in the control treatments described below. Growth experiments were repeated a minimum of three times with consistent results. Data from representative experiments are presented.

For growth experiments with chromate, 50 ml of culture medium containing the indicated concentration of K_2CrO_4 was inoculated with 1 ml of freshly grown inoculum culture and incubated as above.

To study the rate of chromate reduction under conditions that do not favor cell growth, resting cell assays were carried out in the following way: cells were grown overnight in culture medium, harvested by centrifugation, washed twice in 1/4 volume of 10 mM Tris/HCl (pH 7.0) plus 2 mM EDTA and resuspended in 1/2 of this same Tris/EDTA buffer. They were then incubated as above in an orbital incubator and supplemented with the indicated concentrations of K_2CrO_4.

Growth was routinely monitored by measuring optical density at 600 nm (OD_{600}) in an UVIKON 941 Plus Spectrophotometer, Milan, Italy. Growth was also monitored by viable cell counts. Viability was assessed by serial dilutions of culture samples in growth medium lacking chromate, which were immediately plated on the same medium to determine the number of viable cells (c.f.u. ml^{-1}).

Determination of Cr(VI)

The Cr(VI)-reducing activity was assayed spectrophotometrically at 540 nm (with the supernatant fractions obtained by centrifuging samples at 15,000 *x g* for 15 min) by measuring the decrease of Cr(VI) using the diphenylcarbazide reagent (Greenberg et al. 1981). In the experiments with cell-free extracts, and due to the interference caused by some compounds present in the extract itself on the determination of Cr(VI) using diphenylcarbazide (Bopp & Ehrlich 1988), chromate reduction was measured spectrophotometrically. Absorption spectra were obtained for Cr(VI), Cr(III), and the cell extracts showing that chromate had a strong absorbance peak from 350 to 390 nm and that Cr(III) and the cell extracts caused very little interference (although the extracts absorbed in the 350-390 range, they did it to a much lesser extent than chromate). Nonetheless, all blanks were prepared using the extract, thus allowing this interference to be minimized. Chromate reduction was thus quantified by measuring absorbance due to chromate at 375 nm against a reagent blank. When the effect of added NAD(P)H was tested, absorbance was measured at 395 nm in order to minimize interference of NAD(P)H absorbance with that of chromate. The reaction mixture contained 1 ml of extract and 0.5 – 1 mM K_2CrO_4. To study the effect of glucose on chromate reduction in the cell-free extracts, the reaction mixtures were supplemented with 1% (w/v) glucose.

Cell extract

Crude extract was prepared as follows: cells were grown overnight at 30 °C in the culture medium, harvested by centrifugation at 5,000 *x g* and 4 °C, and washed twice (using 1/10 of the initial volume) with 10 mM Tris/HCl (pH 7.0) plus 2 mM EDTA. Subsequently, the cells were resuspended in 1/30 of the initial volume of this same Tris/EDTA buffer and disrupted in an ice bath by two passages through a French pressure cell (SLM Aminco, USA) at 24,000 p.s.i. (165,600 kPa). Unbroken cells were removed by centrifugation at 12,000 *x g* for 10 min at 4 °C. The supernatant was then centrifuged a second time at 32,000 *x g* for 20 min at 4 °C. Finally, the supernatant fluid of this second centrifugation was ultracentrifuged at 150,000 *x g* for 45 min at 4 °C and the new supernatants were dispensed in 2 ml aliquots and immediately frozen at −20 °C until use. The pelleted (membrane) fraction was suspended in the same Tris/EDTA buffer and kept frozen as above. Equivalent amounts of protein of the supernatant fluid (extract) and the resuspended membranes were assayed in all cases.

Protein assay

The protein content was determined according to Peterson (1977) using crystalline bovine serum albumin as standard.

Results

Chromate reduction by whole cells of B. subtilis

To determine whether *B. subtilis* was capable of growth in the presence of chromate, cells grown in chemically defined minimal medium were used as inoculum for this same medium containing 0.5 mM K_2CrO_4. While the control cultures (grown in the absence of Cr) showed no obvious coloration, the cultures grown in the presence of chromate exhibited a yellowish coloration. Similar final levels of growth (maximal cell densities were slightly higher for the control cultures) were observed with and without chromate as determined by OD_{600} and total viable counts, but the growth rates differed, being faster for the control cultures (Figure 1). The data presented in Figure 1 indicated that OD_{600} accurately reflects growth as determined by viable counts. The growth data below are, therefore, given in OD_{600} measurements.

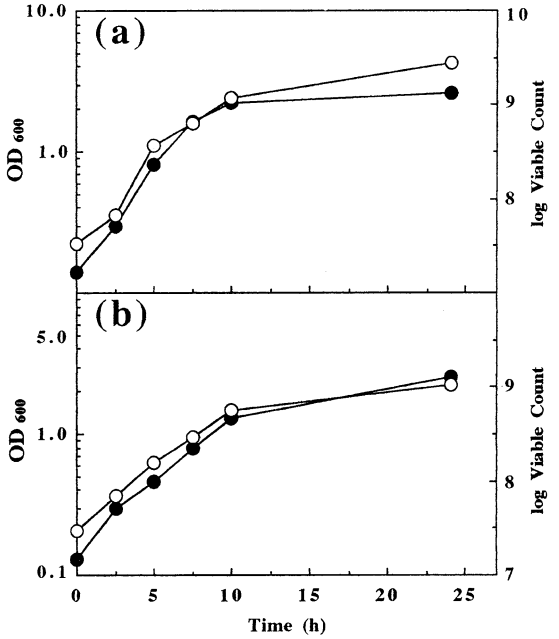

Figure 1. B. subtilis growth expressed as OD_{600} (filled circles) and total viable cell count (empty circles) (c.f.u. ml^{-1}) for **(a)** control (no chromate) culture and **(b)** 0.5 mM K_2CrO_4 culture.

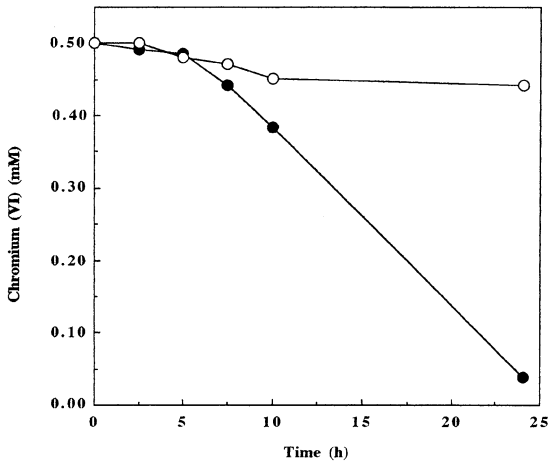

Figure 2. Decrease in Cr(VI) in the supernatant fraction of a culture of *B. subtilis* cells growing in the presence of 0.5 mM K_2CrO_4 (filled circles). The decrease in Cr(VI) was also studied in the absence of cells by means of supplementing the culture medium with the same concentration of chromate (empty circles).

The data above demonstrated that *B. subtilis* could tolerate and grow in the presence of 0.5 mM K_2CrO_4, but left open the question whether chromate was metabolized. This question was answered by analysis of the amount of Cr(VI) remaining in the supernatant fraction of cultures grown in the presence of chromate (Figure 2). As shown in Figure 2, in the presence

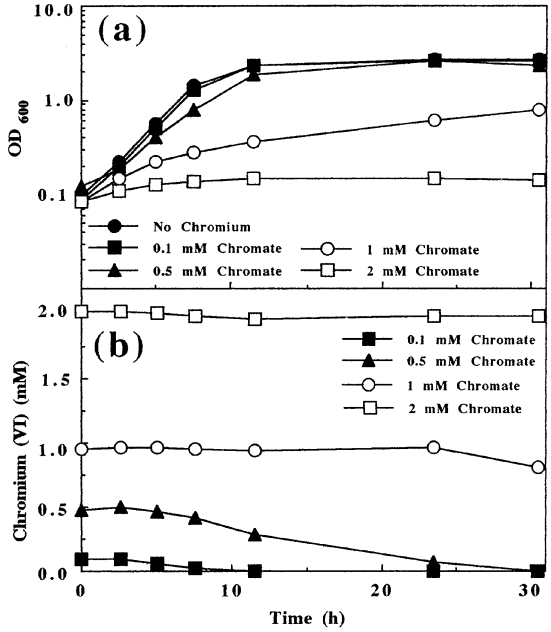

Figure 3. **(a)** Effect of chromate concentration on *B. subtilis* growth. **(b)** Decrease in Cr(VI) in the supernatant fraction of *B. subtilis* cultures supplemented with different chromate concentrations.

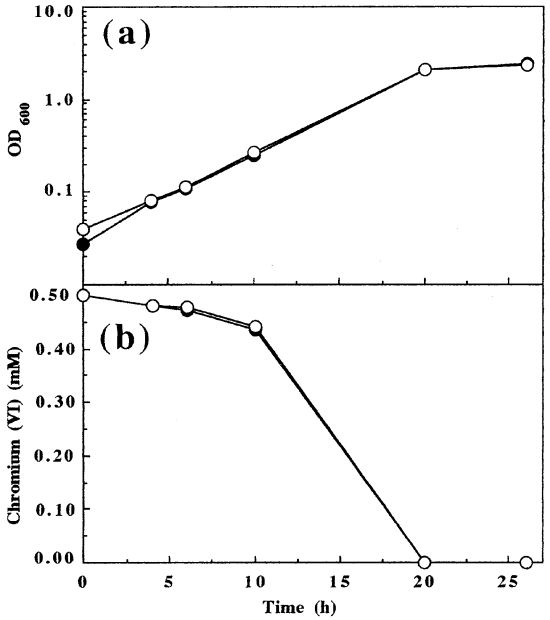

Figure 4. Effect of chromate adaptation on **(a)** *B. subtilis* growth and **(b)** decrease in Cr(VI) in the supernatant fraction of cultures of *B. subtilis* cells growing in the presence of 0.5 mM K_2CrO_4. Non-adapted (filled circles) and adapted (empty circles) cultures were inoculated with a culture grown in the absence or in the presence of 0.5 mM K_2CrO_4, respectively.

of whole cells, the bulk of the Cr(VI) was removed most likely through conversion to the trivalent form. In fact, although a direct demonstration of chromate reduction by means of, for instance, electron paramagnetic resonance spectroscopy was not provided, the reduction of chromates can be judged according to the color change of the medium: whereas at the beginning the medium appears yellow (as expected due to the color of hexavalent chromium), it becomes more and more whitish as the reaction proceeds and chromium hydroxide [$Cr(OH)_3$] is being formed. On the other hand, in the absence of cells (i.e., culture medium supplemented with 0.5 mM K_2CrO_4), there is a certain degree of purely chemical chromate reduction since the amount of Cr(VI) in the medium decreased from 0.50 mM K_2CrO_4 to 0.44 mM after 24 h of incubation. Nonetheless, when cells were present, the decrease in the amount of Cr(VI) was significantly more noticeable (i.e., from 0.50 mM to 0.04 mM after 24 h of incubation) (Figure 2).

Additional experiments revealed that *B. subtilis* has the ability to grow (Figure 3a) and reduce chromate (Figure 3b) in the chemically defined liquid medium at chromate concentrations ranging from 0.1 to 1 mM. Cells failed to grow and reduce chromate at 2 mM chromate. When added at 1 mM, chromate significantly affected cell growth in agreement with the well-known toxicity associated with this form of Cr.

When minimal medium containing 0.5 mM K_2CrO_4 was inoculated with cells previously grown in the same chromate medium, this 'chromate-adapted' culture grew at identical rate than the 'non-adapted' (control) culture (Figure 4a). In addition, both cultures (adapted and non-adapted) followed very similar patterns of chromate reduction (Figure 4b). These results provide evidence that the system effecting chromate reduction is not induced (but constitutive) in *B. subtilis*.

One of the features characterizing anaerobic chromate reduction systems is a sensitivity to nitrate and sulfate ions, both of which compete with chromate (CrO_4^{2-}) as anaerobic electron acceptors. In contrast to anaerobic bacteria, the reduction of chromate by *B. subtilis* was independent of nitrate (since this microorganism does not perform anaerobic sulfate reduction, sulfate was not tested). Neither growth (Figure 5a) nor chromate reduction (Figure 5b) was significantly altered by this oxidant (0.5 mM K_2CrO_4; 10 mM $NaNO_3$). These findings suggest that chromate is not reduced via dissimilatory electron transport in *B. subtilis* but via a detoxification system.

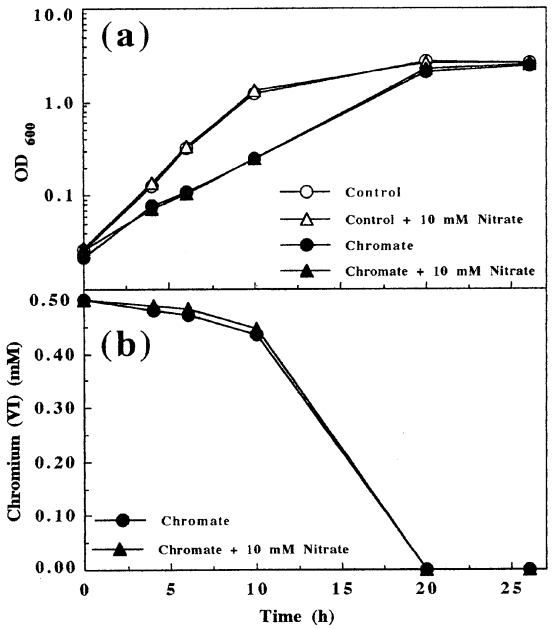

Figure 5. Effect of 10 mM sodium nitrate on **(a)** *B. subtilis* growth and **(b)** decrease in Cr(VI) in the supernatant fraction of cultures of *B. subtilis* cells growing in the presence of 0.5 mM K_2CrO_4 or in the absence of Cr (control cultures), respectively.

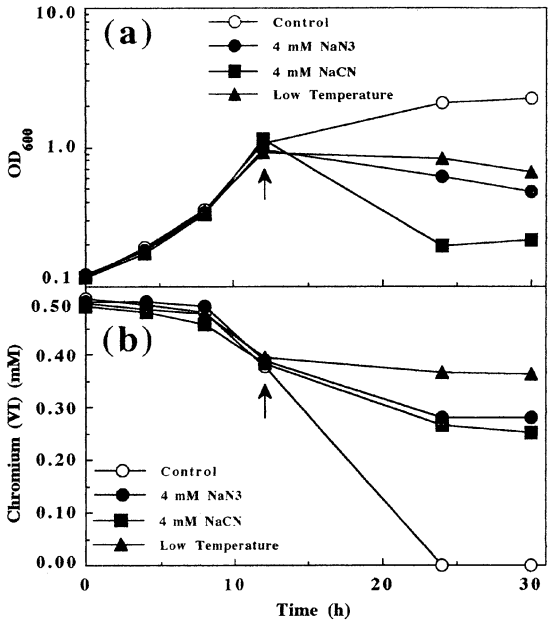

Figure 6. Effect of metabolic poisons (4 mM sodium azide and 4 mM sodium cyanide) and low temperature (4 °C) on **(a)** *B. subtilis* growth and **(b)** decrease in Cr(VI) in the supernatant fraction of cultures of *B. subtilis* cells growing in the presence of 0.5 mM K_2CrO_4. Control cultures were grown in the absence of metabolic poisons and incubated at 30 °C. The cultures were grown under normal incubation conditions till late log phase (arrows) when either the treatment with metabolic poisons or low temperature was applied.

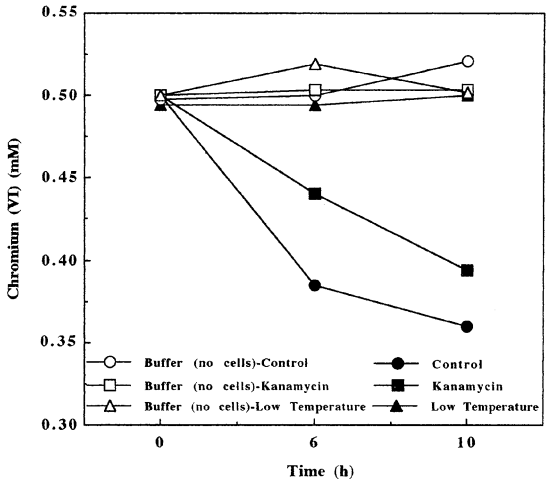

Figure 7. Effect of kanamycin (30 μg ml^{-1}) and low temperature (4 °C) on the decrease in Cr(VI) in the supernatant fraction of cultures of *B. subtilis* resting cells supplemented with 0.5 mM K_2CrO_4. Control cultures were grown in the absence of kanamycin and incubated at 30 °C. In the absence of cells (Tris/EDTA buffer supplemented with 0.5 mM K_2CrO_4), the decrease in Cr(VI) was also determined under the same conditions.

Figure 6 shows the effect of some metabolic poisons (i.e., sodium azide and sodium cyanide) on growth (Figure 6a) and chromate reduction (Figure 6b) of *B. subtilis* cells growing in culture medium supplemented with 0.5 mM K_2CrO_4. In fact, the cells were grown in the absence of these metabolic poisons till late log phase, moment when 4 mM NaN_3/NaCN was added to the flasks. As observed in Figure 6(a), growth was immediately stopped by the addition of these poisons (the control culture, lacking any of these poisons, kept growing till stationary phase). Chromate reduction was also markedly inhibited by these metabolic poisons (Figure 6b). Somewhat different results on growth and chromate reduction were observed when, upon reaching late log phase, the incubation temperature of the cultures was lowered from 30 °C to 4 °C (Figure 6). In fact, the decrease in temperature resulted in an immediate inhibition of the Cr(VI) reduction, whereas the cyanide and azide resulted in only partial inhibition.

To study chromate reduction under conditions which do not favor cell growth, resting cell assays (cells suspended in Tris/EDTA buffer instead of culture medium) were carried out. The effect of the inhibition of the (1) *de novo* protein synthesis as well as (2) metabolic activity on chromate reduction by resting cells was studied by supplementing the cultures with 30 μg kanamycin monosulfate ml^{-1} and incubating

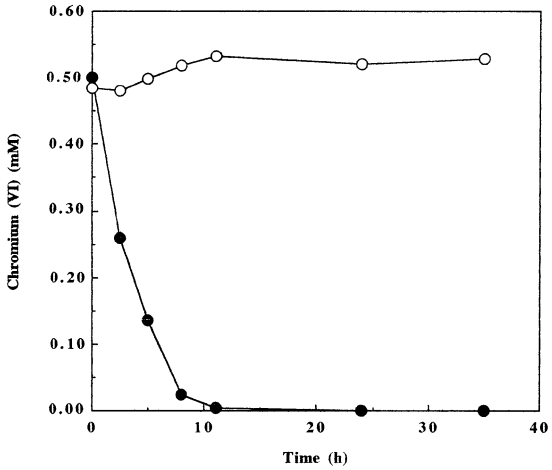

Figure 8. Cr(VI) decrease is associated with the presence of cells. An overnight grown culture was centrifuged to separate the cell fraction from the supernatant fraction. The cells were resuspended in fresh medium and supplemented with 0.5 mM K_2CrO_4 (filled circles). The supernatant fraction was filtered through a 0.45 μm filter and then supplemented with the same chromate concentration (empty circles).

them at 4 °C, respectively. As shown in Figure 7, while incubating the resting cells at 4 °C inhibited chromate reduction, the presence of kanamycin did not stop chromate reduction by resting cells. Nonetheless, the rate of chromate reduction in the kanamycin culture was not as fast as in the control culture (no kanamycin present, and resting cells incubated at 30 °C) (Figure 7). In the absence of cells (Tris/EDTA buffer supplemented with 0.5 mM K_2CrO_4), no chromate reduction could be observed under any of the conditions tested. Similar results were obtained when the resting cell cultures were supplemented with 1% (w/v) glucose (data not shown). However, in this case, the extent of chromate reduction was greater than when the resting cells were incubated in the absence of an external carbon source [Cr(VI) decreased from 0.50 mM to less than 0.15 mM in the kanamycin and control cultures after 10 hours of incubation in the presence of 1% (w/v) glucose] (data not shown).

To confirm that chromate reduction was associated with the cells themselves and not with any possible compounds excreted by the cells to the surrounding medium, after growing them overnight in culture medium, the cultures were centrifuged. While the pellet (cell fraction) was resuspended in fresh culture medium, the supernatant was subsequently filtered under sterile conditions through a 0.45 μm filter (Millipore, Bedford, USA) to remove unpelleted cells and suspended cell debris. Both fractions (cell and supernatant fraction) were then supplemented with 0.5 mM K_2CrO_4. Figure 8 shows that the presence of cells is required for the reduction to occur, since there was no chromate reduction whatsoever in the supernatant filtered fraction.

Table 1. Decrease in Cr(VI) Catalyzed by Cell-Free Extracts of *B. subtilis**

Reaction Time (h)	Soluble Fraction	Boiled Soluble Fraction	Membrane Fraction	Tris/EDTA Buffer
0	100	100	100	100
1	57	100	100	98
2	43	100	100	98
3.5	29	100	99	98
4.5	21	100	98	98

* Values are given as a percentage of initial chromate concentration.
100% corresponds to 0.5 mM K_2CrO_4.

Table 2. Decrease in Cr(VI) Catalyzed by Cell-Free Extracts *of B. subtilis* Supplemented with 1% (w/v) Glucose*

Reaction Time (h)	Soluble Fraction	Boiled Soluble Fraction	Membrane Fraction	Tris/EDTA Buffer
0	100	100	100	100
1	3	100	100	99
2	0	100	99	98
3.5	0	100	99	98
4.5	0	100	99	97

* Values are given as a percentage of initial chromate concentration.
100% corresponds to 0.5 mM K_2CrO_4.

Chromate reduction by cell extracts

With the objective of clarifying the mechanism of Cr(VI) decrease (i.e., chromate reduction) from the liquid phase of the *B. subtilis* cultures, we investigated whether or not a cell free extract was able to decrease the Cr(VI) content as well. As shown in Table 1, cell extracts of *B. subtilis* readily reduced chromate. Actually, the Cr(VI) decreasing activity appears to be located in the soluble fraction obtained after ultracentrifuging the crude cell extracts. No significant reduction was observed with the membrane fractions. Similarly, a soluble fraction heated at 100 °C for 10 min did not cause a decrease in Cr(VI) indicating that the reducing activity was heat labile. The addition of 1% (w/v) glucose to the soluble fraction

Table 3. Effect of NAD(P)H Addition on the Decrease in Cr(VI) Catalyzed by Soluble Cell Fractions of *B. subtilis**

Reaction Time (h)	Soluble Fraction	Soluble Fraction +NADH	Soluble Fraction +NADPH	Tris/EDTA	Tris/EDTA +NADH	Tris/EDTA +NADPH
0	100	100	100	100	100	100
2	99	52	43	98	93	98
4	87	32	27	96	88	96
6	82	27	15	95	85	88
8	57	12	7	90	85	80

* Values are given as a percentage of initial chromate concentration. 100% corresponds to 1.0 mM K_2CrO_4. Cultures were supplemented with 3 mM NAD(P)H as indicated.

caused a dramatic increase in the rate of chromate reduction (Table 2). Nevertheless, no significant decrease in Cr(VI) was found when glucose was added to the Tris/EDTA buffer supplemented with 0.5 mM K_2CrO_4 (3% reduction in 4.5 hours) suggesting that the reduction does not come from the action of glucose itself but some other compound present in the soluble fraction of the cell extracts.

We also examined whether or not redox cofactors had an effect on the Cr(VI) decreasing activity of the extracts. As concluded from Table 3, both NADH and NADPH greatly increased the chromate reducing activity of the soluble fractions. Again, although some reduction was observed when the Tris/EDTA buffer was supplemented with NAD(P)H (around 20% in 8 hours), it was not comparable to that found when the soluble fraction was supplemented with those redox cofactors (around 90% in 8 hours).

Finally, an apparent Michaelis-Menten constant (K_m) of 188 μm for CrO_4^{2-} and a maximum velocity (V_{max}) of 1.25 nmol of Cr(VI) reduced min^{-1} per mg of protein were estimated when data were fitted by non-linear regression to the Michaelis-Menten equation.

Discussion

The present results demonstrate that a well-characterized laboratory strain of the Gram-positive common soil bacterium, *B. subtilis*, removes aerobically hexavalent Cr (chromate) from solution most likely by reduction to the less toxic and more insoluble trivalent form. In fact, whereas at the beginning the culture medium appeared yellow (indicative of the presence of hexavalent Cr), it became more and more whitish as cell growth progressed and theoretically chromium hydroxide [$Cr(OH)_3$] was being formed. Gvozdyak et al. (1986) already described the ability of a facultative anaerobic strain of *B. subtilis* to reduce hexavalent Cr though under anaerobic conditions. In this respect, *B. subtilis* differs from strains previously reported by Kvasnikov et al. (1985), Lebedeva & Lyalikova (1979) and Romanenko & Koren'kov (1977) in its ability to reduce chromate aerobically.

So far, the ability to reduce hexavalent Cr aerobically has been detected in species of the genus *Pseudomonas* (Bopp & Ehrlich 1988; Horitsu et al. 1987; Ishibashi et al. 1990). However, under anaerobic conditions, Gvozdyak et al. (1986) showed that the reduction of Cr(VI) to Cr(III) is not a strictly specific process and can be carried out by many cultures of Gram-positive and Gram-negative microorganisms. In this context, it is worth mentioning the extensive studies carried out with *Enterobacter cloacae* HO1, a strain isolated from activated sludge that uses chromate as an electron acceptor in an anaerobic mode of respiration (Komori et al. 1989, 1990; Ohtake et al. 1990a, b; Wang et al. 1989, 1990, 1991).

Our results support that *B. subtilis* has the ability to grow and reduce chromate aerobically in the chemically defined medium at chromate concentrations ranging from 0.1 to 1 mM while concentrations above those values are lethal to growing cells and prevent the reduction. Other bacteria such as *P. fluorescens* LB300 have been described to tolerate much higher concentrations of chromate and actively reduce Cr(VI) to Cr(III) while growing aerobically in a minimal salts medium (Bopp & Ehrlich 1988). Moreover, *E. cloacae* can reduce chromate at levels of 3–5 mM, although above 5 mM the cells lose viability preventing the reduction as well (Komori et al. 1989).

The *B. subtilis* system of chromate reduction appears to be constitutive. In fact, the lack of significant

effect of kanamycin on the chromate-reducing activity suggests that this activity may not require *de novo* protein synthesis. Similar results were found by Bopp & Ehrlich (1988) in their studies of the rates of chromate reduction by chromate grown cells and cells grown without chromate. In addition, the finding that the reduction of chromate by *B. subtilis* was independent of nitrate suggests that chromate is not reduced via dissimilatory electron transport but via a detoxification system. Similar results have been reported for *P. putida* (Ishibashi et al. 1990).

Metabolic poisons (4 mM of either sodium azide or sodium cyanide) inhibited both cell growth and chromate reduction. Somewhat different results on growth and chromate reduction were observed when, upon reaching late log phase, the incubation temperature of the cultures was lowered from 30 °C to 4 °C. In fact, the decrease in temperature resulted in an immediate inhibition of the Cr(VI) reduction, whereas the cyanide and azide resulted in only partial inhibition. This would be expected as the cyanide and azide prevent *de novo* protein synthesis but do not inhibit the activity of any reductase enzymes that are already present. On the contrary, the reduced temperature will inhibit the activity of any temperature enzymes present in the culture as well as the synthesis of any new enzymes.

Komori et al. (1989) reported that while 100–400 μm sodium cyanide also inhibited chromate reduction in *E. cloacae*, no inhibition was observed with 0.5-1.0 mM sodium azide. However, as indicated, the concentrations used by these authors were much lower than the ones used in our study. From our studies with resting cells, it can be concluded that chromate reduction is not dependent upon cell growth but just on the presence of metabolically active cells.

The reduction by *B. subtilis* appears to be enzymatically-driven, since it is catalyzed by cell extracts. Such chromate reductase activity appeared to be associated with soluble protein rather than with the membrane fraction. Similar results have been reported in *P. putida* (Ishibashi et al. 1990). On the other hand, it has been described (Bopp & Ehrlich 1988) that some or all of the enzymes necessary for the transfer of electrons from NADH to chromate are membrane-bound in a strain of *P. fluorescens* grown aerobically. Wang et al. (1990, 1991) also found that the chromate reductase of the anaerobically grown *E. cloacae* HO1 was preferentially associated with the membrane fraction of the cells.

The chromate reductase in our *B. subtilis* strain is similar to that reported by Horitsu et al. (1987) and Bopp & Ehrlich (1988) in that it can use NADH as electron donor for the reduction of chromate. Nevertheless, although *B. subtilis* can also mediate the transfer of electrons from NAD(P)H to chromate, the reducing enzyme of *P. ambigua* requires NADH but not NADPH. Unlike *P. putida* (Ishibashi et al. 1990), our strain does not require the addition of exogenous NAD(P)H to the cell extract for chromate reduction to occur, since the soluble fraction of the extract itself appears to have the cofactors necessary for the reductase. However, the addition of NAD(P)H significantly enhances the rate of chromate reduction.

B. subtilis also resembles *P. fluorescens* in that it can use exogenous glucose as electron donor too. In this respect, opposite results were found in *P. ambigua* G-1 (Horitsu et al. 1987).

The Cr (VI)-reducing activity was heat labile and showed a K_m of 188 μm for $Cr_2O_4^{2-}$ and a V_{max} of 1.25 nmol of Cr(VI) reduced min^{-1} per mg of protein. Similar results were reported by Ishibashi et al. (1990) in their studies with *P. putida*, although their K_m and V_{max} values were somewhat different (40 μm for $Cr_2O_4^{2-}$ and 6 nmol of Cr(VI) reduced min^{-1} per mg of protein).

These findings raise the possibility that this organism may be useful for environmental restoration of chromate polluted waters and liquid waste treatment. Cr(III) is several orders of magnitude less toxic than Cr(VI) and forms insoluble hydroxides at neutral pH and precipitates, thus making it less bioavailable (Bopp & Ehrlich 1988). *B. subtilis* is able to form dormant spores, which are resistant to heat and dessication, can be easily stored and transported, and readily germinated into vegetative cells by the addition of simple nutrients. These attributes make *B. subtilis* a promising candidate for use in knowledge-based toxic metal remediation systems. Furthermore, and since *B. subtilis* is widely distributed in soils and groundwater systems, the results shown here suggest that these systems have a natural capacity for attenuating chromate pollution.

Experiments are in progress to identify the biochemical mechanisms responsible for reducing chromate in *B. subtilis*. We are currently assessing the proteins involved in the reduction of chromate in this strain in an attempt to unravel the nature of the reductase catalyzing the reduction of Cr(VI) to Cr(III). In this respect, Suzuki et al. (1992) have already reported on the identification of an NAD(P)H-dependent

chromium reductase of *P. ambigua* G-1 that reduces Cr(VI) to Cr(III) with at least two reaction steps via Cr(V) as an intermediate.

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