

Chromate Reduction by Resting Cells of *Agrobacterium radiobacter* EPS-916

SANTIAGO LLOVERA, RAMON BONET, MARIA D. SIMON-PUJOL,
AND FRANCISCO CONGREGADO*

*Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Barcelona,
Avenida Diagonal, 08028 Barcelona, Spain*

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Resting cells of *Agrobacterium radiobacter* EPS-916 grown on glucose, fructose, maltose, lactose, mannitol, or glycerol reduced 0.5 mM chromate. However, resting cells of strain EPS-916 grown on glutamate or succinate did not reduce chromate. The ability of washed cells to reduce chromate was correlated with their redox potential.

Hexavalent chromium is toxic and mutagenic for most organisms (10, 12), and its reduction to less toxic trivalent chromium represents a potentially useful detoxification process (5). Usually, growing cells have been used in detoxification studies (2). However, the activities of nongrowing cells are of fundamental importance in biotechnology, since pregrown immobilized organisms may be used in industrial processes (9). In this paper, we describe chromate reduction by resting cells of *Agrobacterium radiobacter* EPS-916, a strain isolated from soil. This strain was sensitive to chromate under both aerobic and anaerobic conditions and actively reduced 0.05 mM chromate to Cr(III) while it was growing aerobically and 0.15 mM chromate during anaerobic growth with chromate as the electron acceptor (data not shown). However, resting cells of this strain were able to reduce completely concentrations of chromate as high as 0.5 mM.

For resting cell chromate reduction assays, the organism was precultured at 30°C in glucose-mineral salts medium containing 55 mM glucose, 50 mM NH₄Cl, 40 mM KH₂PO₄, 243 μM MgSO₄ · 7H₂O, 2.31 μM MnSO₄, and 4.1 μM CaCl₂ · 2H₂O (pH 7) under both aerobic and anaerobic conditions with either nitrate (25 mM) or chromate (0.15 mM) as the electron acceptor. The cells were removed from the cultures by centrifugation, washed three times with 0.05 M phosphate buffer (pH 7), and resuspended in the same phosphate buffer. A 10-ml portion of this suspension was added to 40 ml of a solution containing potassium chromate at a final concentration of 0.5 mM. The suspension was incubated at 30°C. At intervals, samples were centrifuged, and Cr(VI) and Cr(III) contents were determined in the supernatant by reversed-phase high-performance liquid chromatography as described below. To ensure that all of the chromium was accounted for, the chromium in aliquots of the supernatants was chemically reoxidized by the permanganate azide method (1). Total chromium levels were then compared with the levels in uninoculated controls. More than 95% of the total chromium added initially was always present in the supernatants. Signals of trivalent and hexavalent chromium and their values were determined from aqueous solutions of Cr(VI) as K₂CrO₄ and from aqueous solutions of Cr(III) as CrCl₃. These mixtures were reacted at 60°C for 1 h after EDTA (disodium salt; Sigma) in a Cr-

EDTA mixture (1:50, wt/wt) was added and the pH was adjusted to 5 with 0.1 N HCl (11). The components were fractionated on an RP-18 column (4 by 250 mm) by using 0.2 mM terbuthylammonium in acetonitrile-water (10:90) as the eluent. Column eluates were monitored by measuring the UV absorbance at 250 nm. The elution peak of the Cr(III) chelate was observed at a retention time of 3.1 min. Nonreacted EDTA appeared at 8 to 12 min in a broad, asymmetrical peak. The elution peak of the Cr(VI) species appeared at a retention time of 5 min. Peak areas were plotted as function of concentration. This gave two standard curves, one for Cr(VI) and one for Cr(III)-EDTA; both of these curves exhibited good linearity ($r^2 = 0.9993$ and $r^2 = 0.9970$, respectively). The amounts of Cr(VI) and Cr(III) were determined by using the standard curves. The levels of recovery for all measurements of the total chromium in these peaks ranged from 96.6 to 103.5%. Reproducibility was determined by examining the reactions between EDTA and different concentrations of CrCl₃ and K₂CrO₄ solutions at pH 5. The analytical errors in both Cr(VI) and Cr(III) determinations were within ±3% of the mean values.

Time-related decreases in Cr(VI) concentration accompanied by increases in Cr(III) concentration were observed, indicating that Cr(VI) was reduced by cells pregrown under both aerobic and anaerobic conditions with no electron donor added (Fig. 1). The rate of chromate reduction by resting cells pregrown under anaerobic conditions with NO₃⁻ as the electron acceptor was greater than the rate of chromate reduction by cells pregrown under aerobic conditions. In the absence of cells, no measurable reduction of chromate occurred over a period of several days. This strain may use an endogenous reserve of electron donors, since *A. radiobacter* produces constitutively an intracellular polyglucose storage polymer (7) like that described previously for *Pseudomonas ambigua* G1 (4). The content of intracellular polyglucose was determined as described by Linton and Cripps (6).

Chromate reduction in *A. radiobacter* EPS-916 was observed at pH 5.0 to 8.0 and at 10 to 40°C. The optimum pH was between 7.0 and 7.5, and the optimum temperature was 25 to 30°C. The rate of chromate reduction was dependent on cell density (Fig. 2): the higher the cell density, the greater the reduction rate. Since chromate is a strong oxidizing agent, it was thought that some of the chromate reduction could be a result of a chemical redox reaction between the

* Corresponding author.

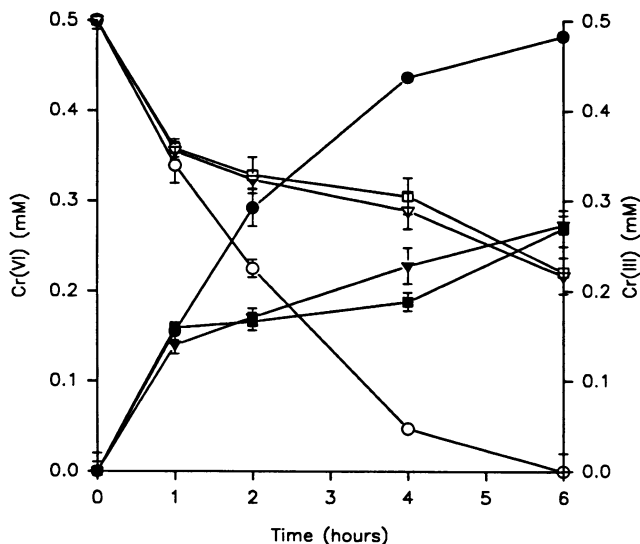


FIG. 1. Comparison of chromate reduction by resting cells of *A. radiobacter* pregrown in glucose-mineral salts medium under aerobic conditions (\square , Cr^{6+} ; \blacksquare , Cr^{3+}) and under anaerobic conditions when CrO_4^{2-} (∇ , Cr^{6+} ; \blacktriangledown , Cr^{3+}) and NO_3^- (\circ , Cr^{6+} ; \bullet , Cr^{3+}) were used as electron acceptors. The inocula contained 2×10^9 cells per ml. The values are the means from three experiments.

bacterial cell organic matter and chromate. However, addition of gramicidine ($20 \mu\text{M}$) to the cellular suspensions resulted in a loss of chromate-reducing activity by the washed cells irrespective of the cell concentration.

The rate of chromate reduction increased with the age of the culture (Fig. 2). The chromate reduction results correlated with changes in the endogenous reserves of the cells (4.5, 15, and 17% [dry weight] intracellular polyglucose for cells that were 24, 48, and 72 h old, respectively).

It has been suggested that some metal-bacterium interactions in resting cells are mediated via cell activities induced during heavy-metal-free pregrowth (8). In prokaryotes most of the reducing power is generated by energy metabolism. Thus, in order to correlate reducing power and chromate reduction, the reducing power was estimated by measuring redox potentials in resting cells of *A. radiobacter* from media containing different carbon and energy sources. The oxidation-reduction potentials of bacterial suspensions in 0.15 M phosphate buffer (pH 7) were measured with an Ingold platinum electrode attached to a digital Beckman Ionalyzer meter. The suspensions were flushed continuously with O_2 -free N_2 . Stable redox potentials were established within seconds while the mixtures were stirred.

Washed cells pregrown under aerobic conditions with glucose, fructose, lactose, maltose, mannitol, or glycerol as the sole carbon and energy source exhibited similar redox potentials (approximately -200 mV), had similar intracellular polyglucose contents (15%, dry weight), and completely reduced chromate ($0.5 \text{ mM CrO}_4^{2-}$ within 6 h). When glutamate or succinate was used as the pregrowth carbon source, the washed cells had a less-electronegative redox potential than the cells pregrown with glucose, fructose, lactose, maltose, mannitol, or glycerol (-138 and -132 mV), and neither chromate reduction nor storage products were detected. Similarly, cells pregrown with glucose as the sole carbon and energy source and nitrate as the electron acceptor under anaerobic conditions exhibited a more-electronegative

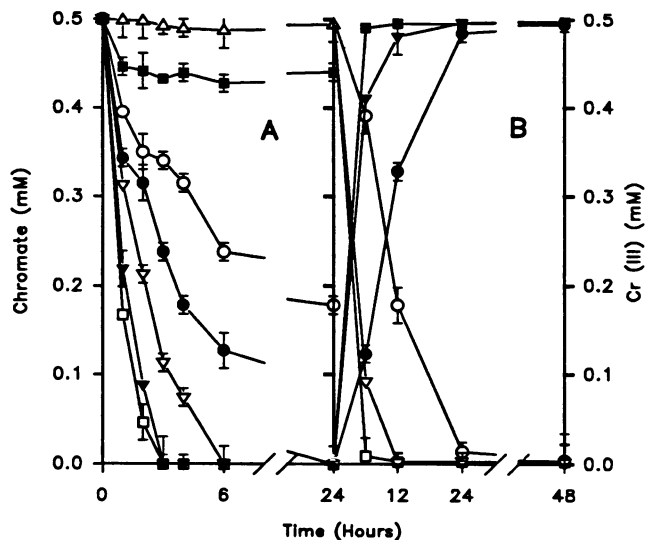


FIG. 2. (A) Effect of viable-cell density on chromate reduction by resting cells. A $0.5 \text{ mM K}_2\text{CrO}_4$ solution was inoculated with various concentrations of *A. radiobacter* EPS-916 pregrown under aerobic conditions. Symbols: \blacksquare , 1×10^5 cells per ml; \circ , 2×10^9 cells per ml; \bullet , 1×10^{10} cells per ml; ∇ , 2×10^{10} cells per ml; \blacktriangledown , 3×10^{10} cells per ml; \square , 4×10^{10} cells per ml without gramicidine; \triangle , 4×10^{10} cells per ml with $20 \mu\text{M}$ gramicidine. (B) Effect of viable-cell age on chromate reduction by resting cells. Washed cells that were pregrown aerobically were resuspended in a 0.5 mM chromate solution at a density of 2×10^{10} cells per ml. Symbols: \circ and \bullet , cells 24 h old; ∇ and \blacktriangledown , cells 48 h old; \square and \blacksquare , cells 72 h old. Open symbols indicate Cr(VI) values, while solid symbols indicate Cr(III) values. All values are the means from three experiments.

redox potential (-240 mV) than cells pregrown in glucose under aerobic conditions and a greater chromate reduction rate (Fig. 1). In this experiment, the more electronegative the cell redox potential was, the greater the chromate reduction rate was. These redox potential differences may have been due to differences in endogenous reserve levels, since intracellular glucose polymer amounts were higher in cells pregrown on carbohydrate than in cells pregrown on succinate or glutamate. However, cells pregrown under anaerobic conditions with either chromate or nitrate as the terminal electron acceptor had similar intracellular polyglucose levels (23 and 22% [dry weight], respectively) and exhibited different chromate reduction rates. These chromate reduction rates correlated well with the redox potentials (-240 and -198 mV , respectively).

Moreover, plasma membrane redox enzymes are a general characteristic of bacterial cells (3) and function to transport reducing power across the membrane. Gramicidine treatment is a common method used to depolarize the membrane; thus, lack of reduction of chromate by *A. radiobacter* cells treated with gramicidine may indicate that chromate is reduced by an active metabolic process related to the energized membrane.

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