Appl Microbiol Biotechnol (1994) 40:756–759

Applied and Microbiology Biotechnology

Reduction of hexavalent chromium by *Pseudomonas fluorescens* LB300 in batch and continuous cultures

Paul C. DeLeo[1]*, **Henry L. Ehrlich**[2]

[1] Department of Civil and Environmental Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180-3590, USA
[2] Department of Biology, Rensselaer Polytechnic Institute, Troy, NY 12180-3590, USA

Received: 1 March 1993/Received revision: 30 July 1993/Accepted: 18 August 1993

Abstract. Batch and continuous cultures of *Pseudomonas fluorescens* LB300 were shown to reduce hexavalent chromium, Cr(VI), aerobically at neutral pH (pH 7.0) with citrate as carbon and energy source. The product of Cr(VI) reduction was previously shown and confirmed in this work to be trivalent chromium, Cr(III), by quantitative reoxidation to Cr(VI) with $KMnO_4$. In separate batch cultures (100 ml) containing initial Cr(VI) concentrations of 314.0, 200.0 and 112.5 mg Cr(VI) L^{-1}, the organism reduced 61%, 69% and 99.7% of the Cr(VI), respectively. In a comparison of stationary and shaken cultures, the organism reduced 81% of Cr(VI) in 147 h in stationary culture and 80% in 122 h in shaken culture. In continuous culture, the organism lowered the influent Cr(VI) concentration by 28% with an 11.7-h residence time, by 39% with a 20.8-h residence time and by 57% with a 38.5-h residence time. A mass balance of chromium in a continuous culture at steady state showed an insignificant uptake of chromium by cells of *P. fluorescens* LB300.

Introduction

Hexavalent chromium, Cr(VI), compounds are widely used in industries such as leather tanning, electroplating and pigment production. Cr(VI) compounds are highly water-soluble and highly toxic, thus necessitating the treatment of waste-water, soils, and sediments containing them. Trivalent chromium, Cr(III), compounds are less soluble and less toxic than Cr(VI) compounds. The health effects of exposure to Cr(III) and Cr(VI) are well characterized (ATSDR 1991; WHO 1988; USEPA 1984). Reduction of Cr(VI) to Cr(III) represents a means by which toxicity is reduced and removal of chromium is facilitated.

Reduction of hexavalent chromium by micro-organisms has been reported under a number of conditions and by a variety of bacterial species. The earliest reports were of an organism isolated from industrial sewage, *Pseudomonas dechromaticans*, which would use chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) as a terminal electron acceptor during anaerobic respiration (Romanenko and Korenkov 1977). Subsequently, a number of organisms have been identified that utilize Cr(VI), generally chromate or dichromate, as a terminal electron acceptor during anaerobic respiration (Lebedeva and Lyalikova 1979; Kvasnikov et al. 1985; Gvozdyak et al. 1986). Anaerobic chromate reduction by *Enterobacter cloacae* HO1 has been exhaustively investigated (Wang et al. 1989, 1990; Komori et al. 1989; Fujii et al. 1990).

Organisms have also been isolated that are able to reduce Cr(VI) under aerobic conditions (Bopp 1980; Horitsu et al. 1987; Ishibashi et al. 1990). *P. fluorescens* LB300 was isolated from chromium-contaminated sediments of the Upper Hudson River, N.Y. (Bopp 1980). The organism demonstrated plasmid-mediated chromate resistance above 1000 mg K_2CrO_4 L^{-1} (Bopp et al. 1983) and was capable of reducing Cr(VI) to Cr(III) aerobically and anaerobically (Bopp and Ehrlich 1988). Under anaerobic conditions *P. fluorescens* LB300 utilized acetate as an electron donor for chromate reduction to trivalent chromium whereas under aerobic conditions the organism used a variety of electron donors for chromate reduction (Bopp and Ehrlich 1988).

The purpose of this study was to demonstrate aerobic Cr(VI) reduction by *P. fluorescens* LB300 with citrate as a carbon and energy source in stationary and shaken batch cultures, and in continuous culture, and to identify some of the factors that may be important in the treatment of Cr(VI) waste with this organism.

Materials and methods

Media. The medium for batch cultures of *P. fluorescens* LB300 consisted of (in g L^{-1}): Bacto-tryptone (Difco, Detroit), 10; yeast

* *Present address:* Department of Soil, Crop and Atmospheric Sciences, Bradfield Hall 1006, Cornell University, Ithaca, NY 14853, USA

Correspondence to: P. C. DeLeo

extract (Difco), 5; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Sigma, St. Louis, Mo., USA) organic buffer, 52; NaCl, 5; and $C_6H_8O_7$ (citric acid), 1. The pH of the medium was adjusted to 7.0 with 2 M H_2SO_4. The medium for continuous-flow cultures consisted of (in g L^{-1}): Bacto-tryptone, 10; yeast extract, 5; NaCl, 5; $C_6H_8O_7$, 1; Na_2HPO_4, 6.9. The ratio of citric acid to disodium phosphate resulted in a buffered medium with pH 7.0.

Stock Cr(VI) solution. A stock solution of Cr(VI) was prepared containing 28.2805 g $K_2Cr_2O_7$ L^{-1} deionized water. Sterilized stock Cr(VI) solution was added to sterile medium to a desired concentration of Cr(VI) with minimal dilution of the medium.

Analyses for Cr(VI) and total chromium. A colorimetric method that utilizes diphenylcarbazide (ASTM 1991) was used to measure Cr(VI) in the supernatant of centrifuged samples from cultures and controls. The total chromium accumulated by cells, in cell wash water, and in the supernatant of culture fluids with cells removed by centrifugation was determined by digesting samples and reoxidizing any Cr(III) to Cr(VI) with potassium permanganate (Ehrlich 1983) followed by the colorimetric analysis for Cr(VI).

The product of bacterial Cr(VI) reduction was inferred to be Cr(III) because it is the only stable, soluble chromium species formed by the reduction of Cr(VI) and because the starting Cr(VI) concentration in culture fluid samples could be restored by chemical oxidation with potassium permanganate (Bopp and Ehrlich 1988; confirmed in this work). Although Cr(II) exists, it is very unstable, readily autooxidizing to Cr(III).

Cell concentration. The optical density of serial 1/8 dilutions of cell suspensions was determined by turbidimetric measurement in a spectrophotometer (Coleman Junior 6A) and calibrated against cell dry weight concentrations. Cell dry weight was determined as described by the American Public Health Association (1985) for total suspended solids.

Batch cultures. Erlenmeyer flasks (250-ml volume) containing 100 ml sterile medium with the desired Cr(VI) concentration were inoculated with 0.1 ml of a cell suspension of *P. fluorescens* LB300 grown in 60 ml medium incubated for 48 h on a rotary shaker at 25° C and 200 rpm. Batch cultures were grown in a stationary mode at 25° C or in a shaken mode at 200 rpm and 25° C.

Continuous-flow cultures. A 50-ml sterile, glass chemostat was used for continuous cultivation at room temperature (approx. 20° C). Flow of the medium and Cr(VI) solution was regulated by a peristaltic pump. The medium and Cr(VI) solution were drawn from separate 500-ml Erlenmeyer flasks containing 300 ml sterile medium and Cr(VI), respectively, at double strength. The feed lines from the medium and Cr(VI) solutions came together after the pump to provide feed solution at the desired composition. Three final flow rates were used: 1.17 ml h^{-1}, 2.26 ml h^{-1}, and 3.85 ml h^{-1}. Sterile air was introduced into the growth vessel with the culture to ensure complete mixing.

Results and discussion

Batch cultures

P. fluorescens LB300 was able to reduce high concentrations of Cr(VI) in a batch culture. Reduction of Cr(VI) was highly linear. Over the period of observation (289 h) Cr(VI) was reduced by 61% in a flask with an initial concentration of 314.0 mg Cr(VI) L^{-1} ($R^2=0.98$), 69% in a flask with an initial concentration of 200.0 mg Cr(VI) L^{-1} ($R^2=0.97$), and 99.7% in a flask with an initial concentration of 112.5 mg Cr(VI) L^{-1} ($R^2=0.98$). The linear pattern followed by the data suggests that the limiting factor in Cr(VI) reduction in the presence of an excess energy source (citrate) was a physical phenomenon such as the diffusion of O_2 rather than Cr(VI) concentration. This was not unexpected, given that the flasks were stationary. The significant residual concentration of Cr(VI) in the two flasks with higher initial Cr(VI) concentrations over the period of observation was also not unexpected. Bopp (1980) demonstrated that at higher Cr(VI) concentrations product inhibition of Cr(VI) reduction by Cr(III) occurred. If these flasks had been incubated further, Cr(VI) reduction in them would have ceased. One can also note that the rate of reduction of Cr(VI) increased with increasing initial Cr(VI) concentration. For the three cases the rate of Cr(VI) reduction was 0.7, 0.5 and 0.4 mg Cr(VI) L^{-1} h^{-1}, respectively.

Cultures of *P. fluorescens* LB300 grown in a shaken mode reduced Cr(VI) at a high rate initially (1.4 mg Cr(VI) L^{-1} h^{-1}), but the rate dropped off very suddenly (0.2 mg Cr(VI) L^{-1} h^{-1}, see Fig. 1). This drop in rate was another example of product inhibition by Cr(III). The initial rate of Cr(VI) reduction in the shaken flask, which had an initial Cr(VI) concentration of 87.2 mg Cr(VI) L^{-1}, was twice that of the stationary flask with an initial Cr(VI) concentration of 314.0 mg Cr(VI) L^{-1}. The higher rate of Cr(VI) reduction may have been part of the reason for observed product inhibition in the shaken flask but other factors may have played a role. It is likely that O_2 availability was a factor. Aerobically, Cr(VI) and O_2 must compete as terminal electron acceptors in a branched respiration pathway. The sudden decrease in the rate of reduction of Cr(VI) in the shaken flask in Fig. 1 could reflect a biochemical advantage of O_2 over chromate at that stage of the growth cycle. In a shaken flask the O_2 con-

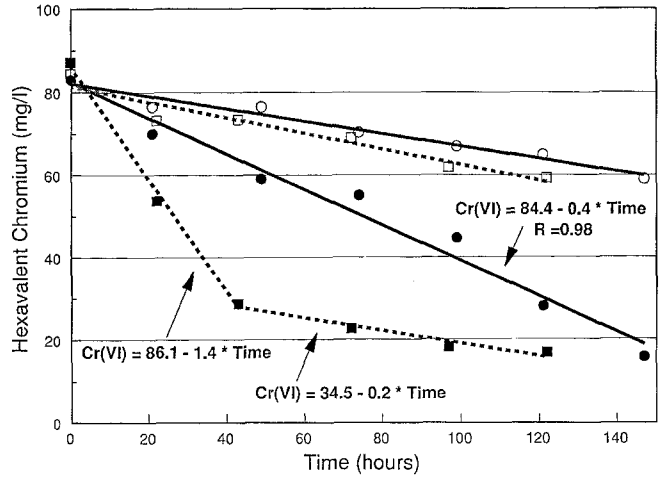

Fig. 1. Reduction of hexavalent chromium, Cr(VI), by a 100-ml batch cultures of *Pseudomonas fluorescens* LB300 in shaken and stationary mode: O—O, sterile control flask in stationary mode; ●—●, inoculated flask in stationary mode; □--□, sterile control flask in shaken mode; ■--■, inoculated flask in shaken mode

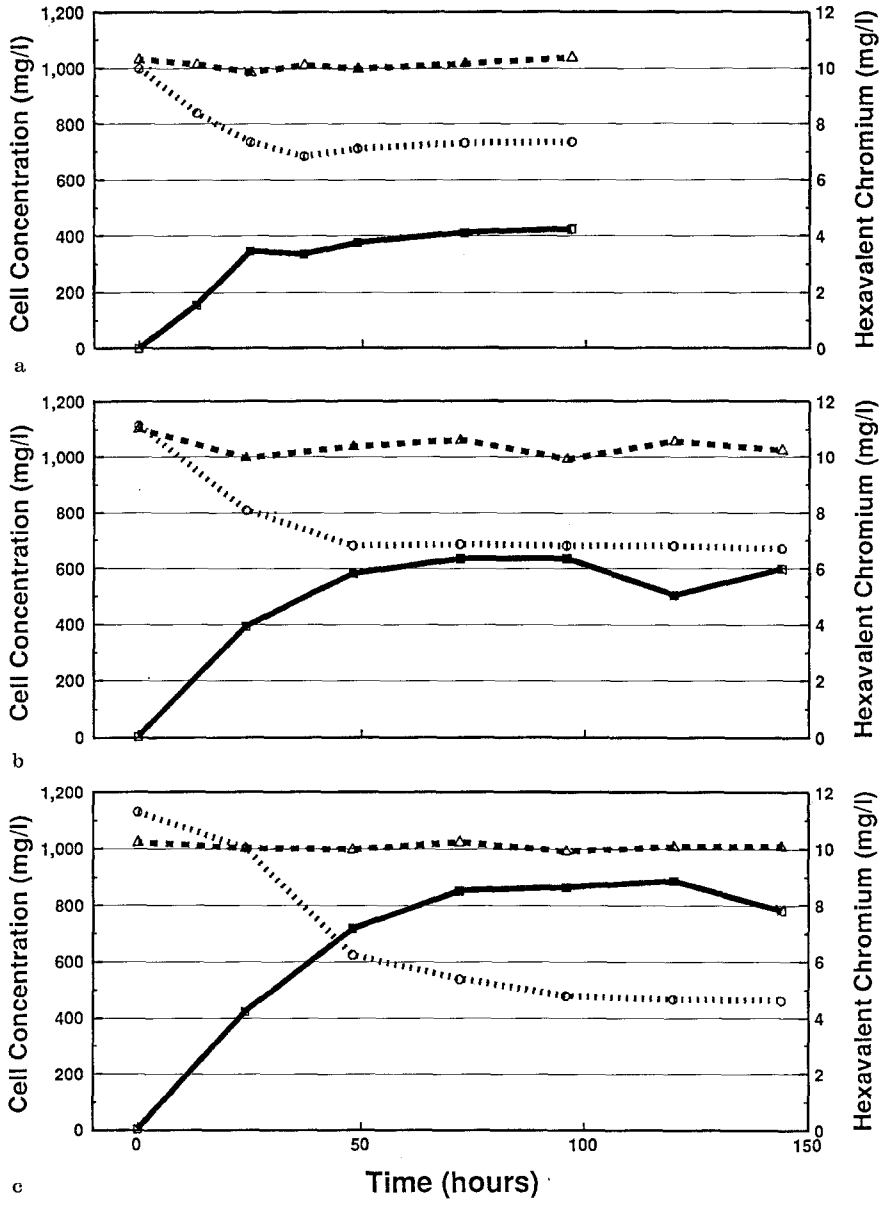

Fig. 2a–c. Reduction of hexavalent chromium in a continuous flow culture at various flow rates in a 50-ml chemostat: **a,** 3.85 ml h^{-1}; **b,** 2.26 ml h^{-1}; **c,** 1.17 ml h^{-1}. △--△, Cr(VI) concentration in sterile control chemostat; ○···○, Cr(VI) concentration in the inoculated chemostat; ■—■, cell concentration in the inoculated chemostat

Table 1. Reduced hexavalent chromium, Cr(VI), and cell concentration as a function of contact time

Contact time (h)	Reduced Cr(VI) (mg l^{-1})	Cell conc (mg l^{-1})	Cr(VI) reduction rate (mg mg^{-1} h^{-1})
11.7	2.57	377	5.83×10^{-4}
20.8	3.64	590	2.97×10^{-4}
38.5	6.27	845	1.93×10^{-4}

centration remains close to saturation, so O_2 would be readily available. In a stationary flask the O_2 concentration within the medium would be limiting to growth so Cr(VI) would continue to be utilized. As suggested by Fig. 1, O_2 was necessary for optimal growth. It became the limiting factor in chromate reduction in stationary cultures due to limited O_2 diffusion.

It is noted that the rate of Cr(VI) reduction in the inoculated stationary culture in Fig. 1 with an initial Cr(VI) concentration of 83.0 mg Cr(VI) L^{-1} was similar (0.4 mg Cr(VI) L^{-1} h^{-1}) to that of the stationary culture in the earlier experiment with an initial Cr(VI) concentration of 112.5 mg Cr(VI) L^{-1}. Also, there was a significant reduction of Cr(VI) in the sterile control flask but the level of reduction in the inoculated flask was several times greater over the same period of observation.

Continuous cultures

Continuous flow cultures showed an increase in Cr(VI) reduction and cell concentration at steady state with increased mean cell residence time (Fig. 2). The increase in cell concentration with longer residence time indicated that citrate was not limiting. The level of ci-

Table 2. Mass balance of chromium in a chemostat at steady state

Sample no.	Initial Cr(VI) in supernatant[a] ($mg\,l^{-1}$)	Final Cr(VI) in supernatant[a] ($mg\,l^{-1}$)	Total Cr in supernatant[a] ($mg\,l^{-1}$)	Cr(III) in supernatant[a,b] ($mg\,l^{-1}$)	Total Cr in cells[a] ($mg\,l^{-1}$)	Total Cr in wash water[a] ($mg\,l^{-1}$)	Cr not accounted for ($mg\,l^{-1}$)
1	10.75	6.75	8.67	1.92	0.15	0.19	1.74
2	10.75	6.58	7.60	1.02	0.17	0.29	2.69
3	10.75	5.52	8.92	3.40	0.23	0.27	1.33

[a] Average of two values
[b] Difference between total Cr in supernatant and final Cr(VI) in supernatant

trate or the dilution rate would have to have been reduced in order for the culture to have been substrate limited.

A comparison of Cr(VI) reduction rate with decreased dilution rate (resulting in increased steady state cell concentration) indicated a decrease in the rate of Cr(VI) reduction per unit mass of cells per unit time (Table 1). This can be interpreted in one of two ways. It may be another indication of inhibition of Cr(VI) reduction by Cr(III) or it may indicate that O_2 is more readily used than Cr(VI) as an electron acceptor under aerobic conditions in the chemostat.

A mass balance of chromium in a chemostat at steady state operated at a flow rate of 2.25 ml h^{-1} revealed that an insignificant amount of chromium was taken up by the cells (Table 2). It also showed that the chromium in solution was present as Cr(III) because it was quantitatively reoxidizable to Cr(VI) (see discussion in Materials and methods). No evidence of $Cr(OH)_3$ precipitate was noted although Cr(III) is reported to be nearly insoluble between pH 7.5 and 9.5 (Parsons 1965), the pH range of the culture in the chemostat. Since citrate, which served as a carbon source and buffer in the medium, was in excess it can be assumed to have chelated Cr(III), keeping it in solution.

These experiments demonstrated that *P. fluorescens* LB300 has a potential for aerobic reduction of Cr(VI) in a batch or continuous process applicable to the treatment of industrial waste streams and bioremediation of contaminated soils, sediments and groundwater. Further research is necessary to optimize the system in terms of substrate concentration, chromate concentration and O_2 concentration. Product inhibition of chromate reduction by trivalent chromium must also be studied further so that it may be minimized.

References

APHA (1985) Standard methods for the examination of water and waste water, 16th edn. American Public Health Association, Washington, D.C.

ASTM (1991) Annual book of ASTM standards, vol 11.01. American Society for Testing and Materials, Philadelphia, Pa., pp 402–404

ATSDR (1991) Toxicological profile for chromium. Agency for Toxic Substances and Disease Registry, U.S. Public Health Service, Atlanta, Ga.

Bopp LH (1980) Chromate resistance and chromate reduction in bacteria. Ph.D. thesis, Rensselaer Polytechnic Institute, Troy, N.Y.

Bopp LH, Ehrlich HL (1988) Chromate resistance and reduction in *Pseudomonas fluorescens* strain LB300. Arch Microbiol 150:426–431

Bopp LH, Chakrabarty A, Ehrlich HL (1983) Chromate resistance plasmid in *Pseudomonas fluorescens*. J Bacteriol 155:1105–1109

Ehrlich HL (1983) Leaching of chromite ore and sulfide matte with dilute sulfuric acid generated by *Thiobacillus thiooxidans* from sulfur. In: Rossi G, Torma AE (eds) Recent progress in biohydrometallurgy. Assoc Mineraria Sarda, Iglesias, Italy, pp 19–42

Fujii E, Toda K, Ohtake H (1990) Bacterial reduction of toxic hexavalent chromium using a batch-fed culture of *Enterobacter cloacae* strain HO1. J Ferment Bioeng 69:365–367

Gvozdyak PI, Mogilevich NF, Ryl'skii AF, Grishchenko NI (1986) Reduction of hexavalent chromium by collection strains of bacteria. Mikrobiologiya 55:770–773

Horitsu H, Futo S, Miyaza Y, Ogai S, Kawai K (1987) Enzymatic reduction of hexavalent chromium by hexavalent chromium tolerant *Pseudomonas fluorescens* G-1. Agric Biol Chem 51:2417–2420

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

Komori K, Rivas A, Toda K, Ohtake H (1990) A method of removal of toxic chromium using dialysis-sac cultures of a chromate-reducing strain of *Enterobacter cloacae*. Appl Microbiol Biotechnol 33:117–119

Kvasnikov EI, Stepanyuk VV, Klyushnikova TM, Serpokrylov NS, Simonova GA, Kasatkina TP, Panchenko LP (1985) A new chromium-reducing, Gram-variable bacterium with mixed type flagellation. Mikrobiologiya 54:83–88

Lebedeva EV, Lyalikova NN (1979) Reduction of crocoite by *Pseudomonas chromatophilia* nov. sp. Mikrobiologiya 48:405–410

Parsons WA (1965) Chemical treatment of sewage and industrial wastes. National Lime Association, Washington, D.C.

Romanenko VI, Korenkov VN (1977) A pure culture of bacteria utilizing chromates and bichromates as hydrogen acceptors in growth under anaerobic conditions. Mikrobiologia 46:329–332

USEPA (1984) Health assessment document for hexavalent chromium. EPA 540-/1-86-019. U.S. Environmental Protection Agency, Cincinnati, Ohio

Wang P, Mori T, Komori K, Sasatsu M, Ohtake H (1989) Isolation and characterization of an *Enterobacter cloacae* strain that reduces hexavalent chromium under anaerobic conditions. Appl Environ Microbiol 55:1665–1669

Wang P, Mori T, Toda K, Ohtake H (1990) Membrane-associated chromate reductase activity from *Enterobacter cloacae*. J Bacteriol 172:1670–1672

WHO (1988) Chromium, environmental health criteria 61. World Health Organization, Geneva