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## Short Communication

# Effect of heavy metals on chromate reduction by *Bacillus subtilis*

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Wastewaters containing chromates [Cr(VI)] are generated in many industrial processes including the manufacture of metallic alloys, ceramics, pyrotechnics, electronics, chrome leather tanning, metal cleaning processing, wood preservation and so on (Cervantes, 1991; Ohtake et al., 1990). Cr(VI), a form of chromium considered hazardous to fauna and flora in natural ecosystems, has been reported to be approximately 100 times more toxic than the relatively innocuous Cr(III) (Luli et al., 1983).

Some bacteria have been shown to reduce chromate to the trivalent form, including strains of *Pseudomonas* (Ishibashi et al., 1990; Suzuki et al., 1992), *Aeromonas* (Kvasnikov et al., 1985), *Enterobacter* (Ohtake et al., 1990; Wang et al., 1991) and other species (Gvozdyak et al., 1986). Chromate reduction occurs both under anaerobic (chromate being used as final electron acceptor) and aerobic conditions. This bacterial capacity to reduce Cr(VI) to the less toxic Cr(III) has potential for the removal of chromates from water.

Unfortunately, chromate-bearing wastewaters from industrial processes generally contain heavy metal cations such as $Cu^{2+}$, $Cd^{2+}$, $Zn^{2+}$ and $Ni^{2+}$ (Germain and Patterson, 1974), which are known to be toxic for most organisms and therefore possibly influence the performance of the chromate-reducing bacteria.

Nonetheless, there is hardly any data published in this respect. In fact, to our knowledge, there is only one report published as to the effects of heavy metals on the anaerobic reduction of chromate by Gram-negative *Enterobacter cloacae* (Hardoyo et al., 1991). Here, we report for the first time, the effect of the most common heavy metal cations found in chromate-bearing wastewaters on chromate reduction by the aerobic Gram-positive bacterium *Bacillus subtilis.*

The organism *B. subtilis* 168t$^+$ (Spanish Type Culture Collection, CECT No. 461) was cultured on a minimal chemically defined liquid medium that contained: 13.9 g/l $K_2HPO_4$, 6.0 g/l $KH_2PO_4$, 2 g/l $(NH_4)_2SO_4$, 1.9 g/l $C_6H_5Na_3O_7 \cdot 2H_2O$, 23.6 mg/l $Ca(NO_3)_2 \cdot 4H_2O$, 19.8 mg/l $MnCl_2$, 0.28 mg/l $FeSO_4 \cdot 7H_2O$ and 1% (w/v) glucose. 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 h. Reinoculations were timed to ensure that cultures were under excess nutrient conditions, and thus in an environment allowing balanced exponential growth. Experiments were repeated a minimum of three times with consistent results. Data from representative experiments are presented.

To study the rate of chromate reduction, cell assays were carried out in the following way: cells were grown overnight in culture medium, harvested by centrifugation, washed twice in 1/10 volume of fresh culture medium and resuspended in 1/4 volume of the same medium. They were then incubated as above in an orbital incubator at 200 rpm and supplemented with 0.5 mM [26 ppm Cr(VI)] $K_2CrO_4$ plus the indicated concentrations of the heavy metal cations (i.e., 0.25, 0.50, 0.75 and 1 mM $CuSO_4 \cdot 5H_2O$, $CdCl_2 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$ and $NiSO_4 \cdot 7H_2O$, respectively).

Similarly, and to study chromate reduction under

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conditions that do not favor cell growth, resting cell assays were carried out by means of substituting the culture medium for either 100 mM Tris/HCl buffer (pH 6.5) or Milli-Q ultrapure water in the above mentioned assays. The concentration of Tris/HCl was rather high (100 mM) so as to avoid the adjustment of pH carried out by the cells under weaker buffering conditions. Previously, the effect of pH on chromate reduction by *B. subtilis* cells suspended in 100 mM Tris/HCl buffer had been determined, showing that 6.5 appears to be the optimum pH value in relation to the chromate reducing capacity of the cells (data not shown).

At this point, it should be indicated that, due to the high variability found in industrial wastewaters regarding their different physicochemical characteristics (pH, temperature, chemical composition, etc.), we decided not to perform chromate reduction experiments using any chromate-bearing industrial wastewater. We believe that the results would be highly specific to that particular effluent, not providing data of general interest.

Growth was routinely monitored by measuring absorbance at 600 nm in a UVIKON 941 Plus Spectrophotometer, Milan, Italy. The Cr(VI)-reducing activity was assayed spectrophotometrically at 540 nm (with the supernatant fractions obtained by centrifuging samples at 15,000×*g* for 15 min) by measuring the decrease in Cr(VI) using the diphenylcarbazide reagent (Greenberg et al., 1981).

Initially, the decrease in Cr(VI) in the supernatant fraction of cultures incubated without added metal cations was studied. As shown in Fig. 1, the rate of chromate reduction of cells growing in culture medium was much faster than those found with resting cells (cells suspended in either Tris/HCl buffer or Milli-Q water). 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: at the beginning, the medium appears yellow (as expected due to the color of hexavalent chromium) and it became more and more whitish as the reaction proceeds and chromium hydroxide [$Cr(OH)_3$] is formed. In the absence of cells, the control flasks (culture medium/Tris buffer/Milli-Q water supplemented with 0.5 mM $K_2CrO_4$) showed no significant reduction of chromate (data not shown).

Since there was hardly any chromate reduction in those cultures containing cells suspended in Milli-Q water, no more experiments were carried out under these conditions. However, when cells were suspended in 100 mM Tris/HCl buffer, pH 6.5, there was a significant decrease in Cr(VI) in the supernatant fraction (Fig. 1).

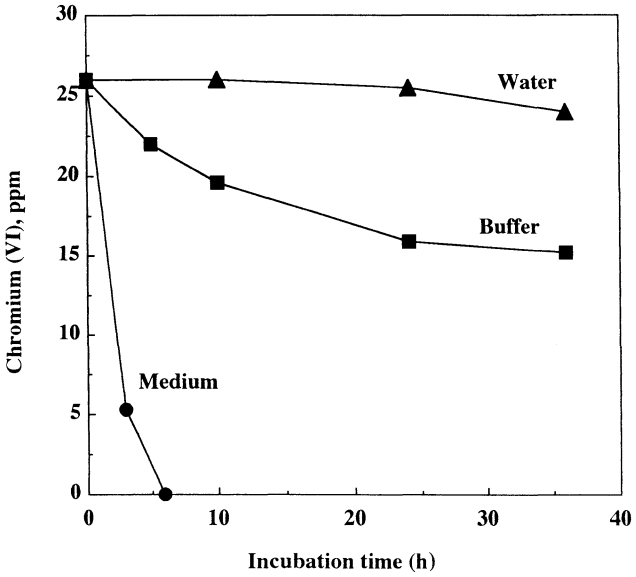


Fig. 1. Chromate reduction.
Decrease in Cr(VI) in the supernatant fraction of *B. subtilis* cells suspended in culture medium, 100 mM Tris/HCl buffer, pH 6.5, and Milli-Q water. Cultures were supplemented with 0.5 mM $K_2CrO_4$ [26 ppm Cr(VI)].

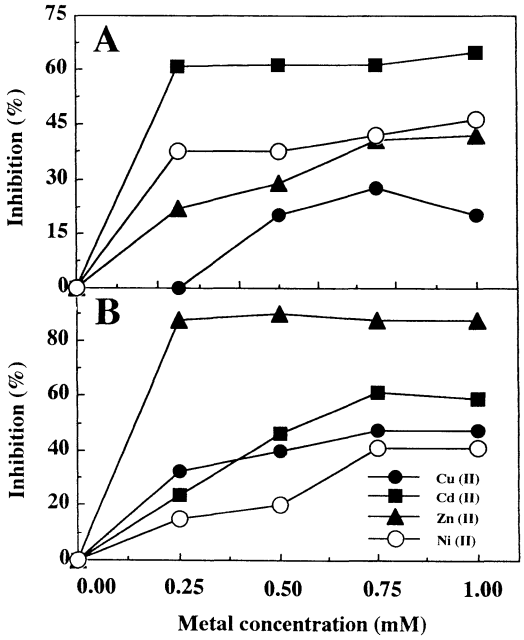


Fig. 2. Effect of heavy metals on chromate reduction.
Effect of heavy metal cations on chromate reduction (expressed as percent inhibition) by *B. subtilis* cells suspended in (A) culture medium and (B) 100 mM Tris/HCl buffer, pH 6.5.

As observed in Fig. 2, all of the heavy metals tested inhibited chromate reduction by *B. subtilis* cells suspended in either culture medium (Fig. 2A) or 100 mM Tris/HCl buffer (Fig. 2B). The percent inhibition was calculated from the initial rate of chromate reduction relative to that of the control culture without added

metal cations. As expected, the higher the concentration of metal used the higher the percent inhibition of chromate reduction. The pattern of inhibition caused by the tested metal cations varied depending on whether the cells were suspended in culture medium (where the metal cations most likely interact with the other ions present, probably modifying their bioavailability) or Tris/HCl buffer. The degrees of inhibition caused by the metal cations were $Cd^{2+}>Ni^{2+}>Zn^{2+}>Cu^{2+}$ and $Zn^{2+}>Cd^{2+}>Cu^{2+}>Ni^{2+}$ for the cells suspended in culture medium and Tris/HCl buffer, respectively.

Cells growing in culture medium supplemented with different concentrations of metals showed faster rates of chromate reduction than the less metabolically active resting cells suspended in Tris/HCl buffer (Fig. 2). At this point, it should be noted that biomass growth in the cultures containing cells suspended in culture medium was very limited and similar in all concentrations and metals tested (data not shown), most likely due to the high cell densities (values of absorbance at 600 nm were around 1.6) and short experimental times (6 h) used in our studies. It is expected that chromate reduction rates would change under different growth conditions.

To our knowledge, this is the first report of a study on the inhibitory effects of heavy metal cations on aerobic chromate reduction by Gram-positive bacteria. However, similar results indicating the strong inhibition caused by heavy metal cations on the reduction of chromate were reported by Hardoyo et al. (1991) for anaerobically grown *E. cloacae. B. subtilis* cells appear to be significantly more resistant to the presence of these metal cations, as derived from the lower values of percent inhibition of chromate reduction found in our studies in comparison with those reported by Hardoyo et al. (1991).

Our results indicate that, although *B. subtilis* cells can still reduce Cr(VI) in the presence of different heavy metal cations, the performance of a potential biological treatment of chromate-bearing wastewaters would be significantly improved by removing toxic heavy metal cations from industrial effluent. Unfortunately, conventional methods for removing metals from effluents (ion exchange, adsorption on coal, activated carbon, etc.) are expensive due to their requirements for high energy or large quantities of chemical adsorbents. In this context, the removal of heavy metals by microbial cells, mainly through their sorption to cell surfaces, has been recognized as a potential alternative to the existing technologies for the recovery of metals from industrial wastewaters (Sampedro et al., 1995). Additionally, if the concentrations of metals in the effluent are low, appropriate dilution could help to overcome their inhibition effect on the biological reduction of chromate.

Finally, much research is needed to unravel the specific inhibition mechanisms for each heavy metal.

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