

Toxicity of Cr(III) to *Shewanella* sp. Strain MR-4 during Cr(VI) Reduction

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Bioremediation of chromium through the reduction of hexavalent chromium (as the chromate ion, CrO_4^{2-}) is based on the notion that the product, trivalent chromium (Cr(III)), is less toxic than chromate. In this study, we show that soluble Cr(III), present at pH 6–8 as the Cr^{3+} ion and/or hydroxyl complexes (henceforth referred to as uncomplexed Cr(III)), can be found transiently in significant concentrations and has a deleterious effect on *Shewanella* sp. MR-4. However, Cr(III) complexed to an organic ligand or precipitated as $\text{Cr}(\text{OH})_3(\text{s})$ has little or no effect on cells. Similarly, during the reduction of Cr(VI) by strain MR-4, complexation of the product, Cr(III), results in increased cell survival and extended Cr(VI) reduction activity. These results and gene expression data obtained by qRT-PCR (quantitative reverse transcription-PCR) suggest that the observed toxic effect of Cr(III) formed during Cr(VI) reduction or added as an uncomplexed species is due to the interference with basic cell activities such as DNA transcription and/or replication. Important implications for the bioremediation of Cr(VI)-contaminated sites emerge from this study: Cr(VI) reduction by *Shewanella* sp. MR-4 is enhanced and sustained by the presence of compounds able to complex Cr(III) as it is being formed but, in turn, the complexation of Cr(III) precludes its precipitation and immobilization.

Introduction

Chromium contamination is ubiquitous as a result of industrial activities such as ship-building, metallurgy and leather tanning. As an environmental contaminant, chromium is found mostly in its oxidized, hexavalent form. Bacterial reduction of hexavalent chromium (as the chromate ion, CrO_4^{2-}) is considered one of the promising strategies for the bioremediation of chromium contamination. The process

is based on the notion that hexavalent chromium is a toxic (I), soluble species that moves fairly rapidly in the subsurface and that can readily enter a cell, whereas the reduced form, trivalent chromium, is relatively insoluble and immobile, and thus not bioavailable and nontoxic (I). Thus, the in situ reduction of Cr(VI) to Cr(III) would, in principle, lead to the immobilization of the waste and its simultaneous detoxification.

In the environment, Cr(VI) may be reduced enzymatically by a phylogenetically diverse group of microorganisms which includes sulfate-reducing bacteria (SRB) and iron-reducing bacteria (IRB). In addition, Cr(VI) is reduced chemically by hydrogen sulfide or ferrous iron which are end products of sulfate or iron reduction respectively. However, recent studies (2, 3) have documented that SRB are sensitive to Cr(VI) and, consequently, their role in Cr(VI) reduction is limited. In contrast, iron-reducing bacteria such as *Shewanella* spp. may be important for the enzymatic and/or chemical reduction of Cr(VI) as representatives of that genus were found in enrichment cultures from Cr(VI)-contaminated sites (2). Little is known about the effect of Cr(VI) or Cr(III) on the survival of these bacteria.

In a previous study, we found that after an initial period of chromate reduction by *Shewanella oneidensis* MR-1, the activity stopped (4). In other words, only a limited amount of Cr(VI) could be reduced per cell of strain MR-1 under both aerobic and anaerobic conditions (4). This indicates that the accumulation of reduced chromium may be deleterious to cells. There was no evidence that Cr(VI) could serve as an electron acceptor for anaerobic growth. Furthermore, reduced Cr was identified both extracellularly and in the cytoplasm of cells (4).

This work focuses on investigating the mechanism by which chromium causes the observed deleterious effect on *Shewanella* spp. cells. This report is centered around *Shewanella* sp. MR-4 which, unlike strain MR-1, is capable of anaerobic growth with Cr(VI) as the sole electron acceptor when Cr(VI) is added repeatedly at low concentrations. We found that the hexavalent chromium was not toxic to cells but that the product of Cr(VI) reduction, trivalent Cr – Cr(III) – jeopardizes the survival of cells when found transiently in an uncomplexed form (as the Cr^{3+} ion and/or hydroxyl complexes). In contrast, Cr(III) complexed by an organic or bound in an aged mineral ($\text{Cr}(\text{OH})_3(\text{s})$) has no effect on cell survival. We conclude that the transient uncomplexed Cr(III) formed during Cr(VI) reduction enters the cells through an as-yet unknown mechanism and interferes with basic cell processes.

The implication of these results for the bioremediation of Cr(VI)-contaminated aquifers is that facilitating the complexation of Cr(III) as it is produced during Cr(VI) reduction would enhance the reduction process and sustain it for a longer period of time. However, complexation would also result in the formation of a soluble Cr(III) which may be mobile and whose propensity to dissociate and precipitate needs to be evaluated.

Experimental Section

Bacterial Strain and Culture Conditions. *Shewanella* sp. strain MR-4, used in this study, was isolated from the Black Sea water column by Dr. K. Nealson (5) and stored as a freezer stock at -80°C in our laboratory culture collection until use. Strain MR-4 serves as a model organism for iron-reducing bacteria in marine and freshwater environments as, even though it was isolated from a marine environment, cells grow well in a minimal medium described previously (6), designed

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for the freshwater bacterium *S. oneidensis* MR1 and called shewanella medium (SM). Unless otherwise indicated, SM was always amended with lactate (20 mM) and bicarbonate (30 mM).

Cr(VI) Reduction Time Course Experiments. An inoculum of strain MR-4 cells was grown aerobically at room temperature overnight in 5 mL of SM and 1 mL was used to inoculate 100 mL of SM amended with 100 μ M chromate and either 30 mM Tris (Tris (hydroxymethyl) aminomethane hydrochloride) or 30 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer, both at pH 8 in 250 mL Erlenmeyer flasks. The cultures were grown under nominally aerobic conditions by rotary shaking at 30 °C at ~150 rpm. Measurement of the dissolved oxygen (DO) concentration by a sulfide insensitive Clark type oxygen electrode (Strathkelvin Instruments, Glasgow) indicated that the DO was depleted when cells reached late exponential phase. Cell growth was measured over time by optical density at 600 nm (OD_{600}). Cr(VI) in solution was determined colorimetrically with diphenyl carbazide (DPC) after filtration through a 0.22 μ m filter (7). When Cr(VI) was found to be below detection during growth, an additional 100 μ M Cr(VI) was added. Dissolved Cr was analyzed by inductively coupled plasma–optical emission spectrometry (ICP–OES) after filtration through a 0.22 μ m filter (Pall Acrodisc HT Tuffryn membrane, New York) or in some cases a 0.02 μ m filter (Anotop 10, Whatman, Florham Park, NJ). Cr(VI) reduced at a given time point was determined by subtraction of the amount of Cr(VI) measured in solution at that time point from that measured initially. The presence of soluble Cr(III) was calculated as the difference between total Cr that passed through the filters (determined by ICP–OES) and that which reacted with DPC (i.e., Cr(VI)). Precipitated (particulate) Cr(III) was computed from the difference between total Cr(VI) reduced and soluble Cr(III). Comparison of soluble Cr after filtration through 0.2 μ m versus 0.02 μ m filters and before and after ultracentrifugation at 20 000 \times g for 1.5 h indicated that colloidal Cr species were unimportant except in the case of freshly precipitated Cr(OH)₃(s). Consequently, we used 0.02 μ m filters only for the latter case.

Effect of Complexing Agents on Cr(VI) Reduction. In addition to the time-course experiments described above, end-point experiments were performed to evaluate the effect of 30 mM bicarbonate, 1% humic acid (Leonardite standard humic acid from the International Humic Substances Society), 5 mM citrate (sodium citrate, Sigma) or 5 mM DFO (Desferrioxamine mesylate, Sigma) on Cr(VI) reduction. For these experiments, strain MR-4 cells were grown overnight in SM buffered only with 30 mM bicarbonate at pH 8. Cells were harvested, resuspended in SM with lactate added, and equally distributed among flasks containing 100 μ M Cr(VI) and one of the above-mentioned compounds. In the flasks where Cr(VI) was completely reduced, 100 μ M Cr(VI) was added sequentially until reduction stopped. Plate counts did not show any growth during the duration of the experiment (a few hours).

Preparation of Cr(III) Species. The effect of various species of Cr(III) (Cr–DFO, Cr–Tris, and freshly precipitated or aged Cr(OH)₃(s)) on the survival of strain MR-4 cells was evaluated. Complexes of Cr(III) with DFO or Tris were formed by adding either 100 mM DFO or 600 mM Tris to 5 mL of a solution of 1 M CrCl₃(s) in MQ water. After equilibration for 2–3 h, 95 mL of SM lacking lactate was added to the mixtures. The pH was slowly adjusted to ~8 by addition of 0.1 N NaOH. As the pH was raised, uncomplexed Cr(III) precipitated as Cr(OH)₃(s). Throughout this manuscript, uncomplexed Cr(III) refers to the free Cr³⁺ cation and any soluble hydroxyl species of Cr³⁺. The solutions were left to equilibrate overnight before centrifugation to remove the precipitate and filtration through 0.22 μ m filters to sterilize. The resulting solution was SM

with soluble Cr(III) at a concentration of 613 μ M or 204 μ M complexed by Tris or DFO, respectively. Aged Cr(OH)₃(s) was formed by the addition of a final concentration of 50 mM CrCl₃(s) to 100 mL of sterile SM lacking lactate and adjusted to pH 8. The resulting ~50 mM of Cr(OH)₃(s) precipitate was aged for 4 days before use. Freshly precipitated Cr(OH)₃(s) was prepared by adding either 500, 20, or 10 μ L of sterile 0.5M CrCl₃ to 100 mL of sterile modified SM (MSM). MSM was used for all experiments involving freshly precipitated Cr(III) because it was found to have less propensity to complex Cr(III) than SM and contains 45 mM MES (2-(N-morpholino)ethanesulfonic acid) as the buffer, 1% galactose as a carbon source instead of lactate, no bicarbonate and pH was adjusted to 6 instead of 8. Freshly precipitated Cr(OH)₃(s) was used within minutes of preparation.

Cells were grown in 300 mL of SM or MSM overnight, centrifuged and resuspended in 60 mL of fresh SM or MSM. 5 mL of cell culture was added to each of two replicates containing 25 mL of SM containing Cr–DFO, Cr–Tris, or aged Cr(OH)₃(s) or 25 mL of MSM containing freshly precipitated Cr(OH)₃(s) prepared as described above. The third replicate was used as a no cell control. Prior to cell addition, solution Cr was measured by ICP–OES after filtration through a 0.22 μ m filter (or 0.02 μ m filter for freshly precipitated Cr(OH)₃(s)).

Cell Survival. After reduction stopped, total soluble Cr and remaining Cr(VI) were measured and cells were diluted 10⁵-fold or 10⁶-fold in SM or MSM and 100 μ L of the dilution plated on LB plates in triplicate to evaluate cell survival. Cell survival was determined by counting colony-forming units (CFU) on LB agar plates plated immediately after cell addition and at regular time intervals thereafter. The plates were incubated at 30 °C overnight before counting was carried out.

Transmission Electron Microscopy. Strain MR-4 was grown as before in SM containing 100 μ M Cr(VI) and 30 mM Tris or 30 mM HEPES. An additional 100 μ M Cr(VI) was added to the Tris culture after complete reduction of the initial Cr(VI). After 20 h of growth, the cells were centrifuged and the supernatant discarded. Bacterial cells were processed as described previously (4) with the difference that Spurr's resin was used and that the sections were examined by a JEOL 2000FX II transmission electron microscope (TEM) operated at 200 kV. Images (2048 \times 2048 pixel) were acquired using a Tietz TemCam F224 CCD camera.

qRT–PCR. Quantitative reverse transcription–polymerase chain reaction (qRT–PCR) was utilized to assess how Cr(III) affects the expression of genes encoding proteins directly involved in basic cell processes such as DNA replication. We chose DNA polymerase I (*polA*) and three subunits of DNA polymerase III (*holA*, *dnaE*, *dnaN*) as such indicator genes. The expression of three of the four genes was evaluated in the presence of Cr(III) as compared to its absence. The expression of *polA* was also investigated in the presence of Cr(III) complexed to Tris or when Cr(VI) was being reduced with or without Tris.

Strain MR-4 cells were grown overnight under four separate conditions: in the presence of 0.1 mM Cr(VI), with or without 30 mM Tris; in the presence of Tris but no Cr(VI); and in the absence of both Cr(VI) and Tris. RNA was preserved in the suspensions by addition of a final concentration of 4M guanidine isothiocyanate (GTC) to 20 mL decanted into a 50 mL falcon tube. GTC allows the preservation of RNA immediately upon its addition, minimizing the effect of centrifugation on RNA production and degradation. Cells were harvested by centrifuging 1.5 mL of culture, discarding the supernatant and centrifuging an additional 1.5 mL in the same tube. After discarding the supernatant the second time, 1 mL of TRIzol Reagent (Gibco BRL, Gaithersburg, MD) was

added immediately. The pellet was resuspended and the samples frozen at -80°C until RNA extraction.

Subsequently, the culture containing neither Cr(VI) nor Tris was centrifuged under sterile conditions and resuspended in 10 mL of sterile SM medium and the same volume of cell suspension added to three flasks: one contained 0.8 mM of preformed Cr–Tris complex in SM, the second contained freshly precipitated 1.5 mM Cr(OH)₃(s) in SM, and the third contained only SM. After 30 min, cells were harvested as above.

Total cellular RNA was isolated as described previously (6). cDNA for a given primer set was synthesized from the extracted RNA with the reverse primer designed based on strain MR-1's genome (Table 1, Supporting Information) using Superscript RT II (Gibco, BRL). cDNA was used as a template for quantitative PCR (qPCR) using a MX3000P real-time PCR system (Stratagene, La Jolla, CA), the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA), and 10 μM of each primer (reverse and forward). Strain MR-4 genomic DNA (gDNA) was used as a standard for qPCR. The DNA dissociation curves were identical for gDNA and cDNA indicating that the same sequences were amplified. Results gave a relative concentration of cDNA in each of the samples that allowed the quantification of the change in expression after chromium addition.

Results and Discussion

Effect of Complexing Agents on Cr(VI) Reduction. The presence of Cr(VI) does not appear to affect the growth of strain MR-4 (either with Tris or Hepes as a buffer) for the first 9 h (Figure 1A). The effect of Cr(VI) on growth, which is especially pronounced for the case of Hepes, becomes apparent at ca. 14 h for Tris and 10 h for Hepes (Figure 1A), only after the onset of reduction (which is after 6 h for Tris and 10 h for Hepes, Figure 1B). This observation suggests that the chromate ion is not toxic to strain MR-4 cells per se but that the product of the reduction (Cr(III)) is. In addition, strain MR-4 cells grew much better in the presence of 30 mM Tris vs 30 mM Hepes (Figure 1A) and were able to reduce twice as much Cr(VI) when normalized to a per cell basis (Figure 1B). Thus, Tris promotes Cr(VI) reduction by strain MR-4. Measurement of solution Cr(III) during Cr(VI) reduction as indicated by the difference between DPC-reactive Cr and Cr that passes through a 0.22 μm filter and measured by ICP–OES shows that, in the presence of Tris, almost all the reduced Cr (i.e., Cr(III)) remained in solution up to ~ 14 h (Figure 1B) instead of precipitating as would be expected at pH 8. A similar phenomenon is observed for strain MR-1 (data not shown). Conceivably, reduced Cr is retained in solution by complexation. The system containing Hepes, otherwise identical to the Tris system, shows significant Cr(III) precipitation as well as considerably less Cr(VI) reduction (Figure 1B). This indicates that Tris is the complexing agent retaining Cr(III) in solution in the former case. Tris is widely used as a buffer and is well-known to complex divalent cations and, to some extent, trivalent metal ions.

One explanation for the observed behavior is that the concentration of Tris (30 mM) is in great excess of that of Cr(III) (~ 300 fold excess initially and ~ 100 fold after 24 h) which drives the equilibrium toward complexation even if the affinity of the metal for the ligand (formation constant) is not very large. The actual value of the equilibrium constant for Cr(III)-Tris complex is not known. Electron paramagnetic resonance (EPR) analysis of the solution confirmed that the solution Cr was indeed Cr(III) (data not shown). At 14 h, after ~ 320 μM Cr(III) is complexed in solution by Tris, the concentrations of total Cr(VI) reduced and soluble Cr(III) start to deviate (Figure 1B), indicating the onset of precipitation. Concomitant with precipitation, Cr(VI) reduction ceases despite the presence of ~ 50 μM Cr(VI) in solution. In contrast,

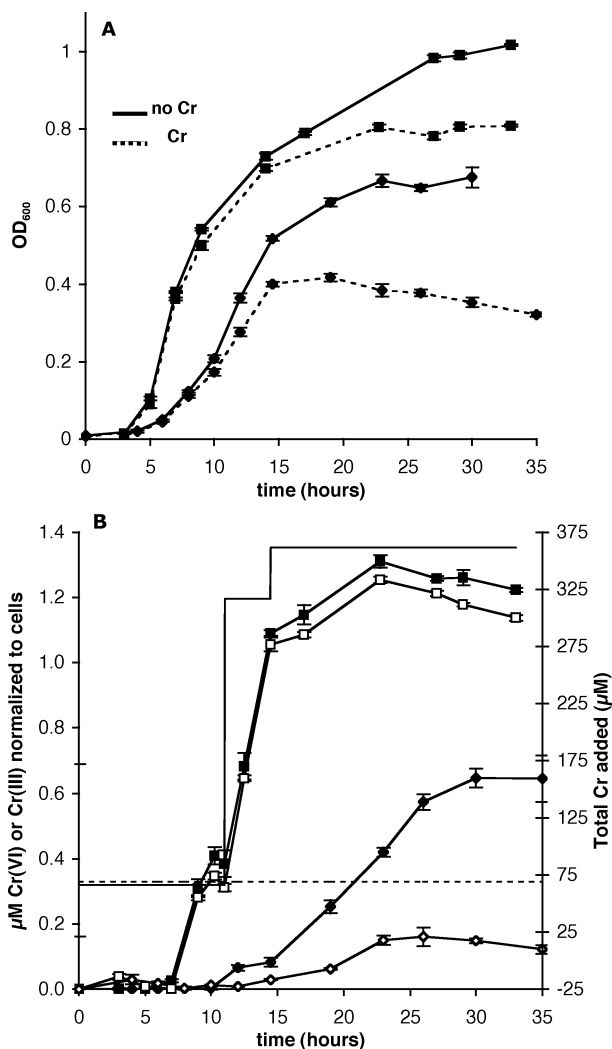


FIGURE 1. A. Growth of strain MR-4 in the presence (----) and absence (—) of Cr(VI) with either 30 mM Tris (■) or 30 mM Hepes (◆) as the buffer. **B.** Cr(VI) reduced by growing strain MR-4 cells in the presence of 30 mM Tris (■) and the corresponding dissolved Cr(III) (□). Similarly, Cr(VI) reduced in the presence of 30 mM Hepes (◆) and the corresponding dissolved Cr(III) (◇). Both Cr(VI) reduced and dissolved Cr(III) are normalized to the number of cells. Total Cr(VI) added to the 30 mM Tris medium (—) and to the 30 mM Hepes medium (----).

in the Hepes culture, Cr(III) precipitates almost at the onset of Cr(VI) reduction (~ 10 h), as evidenced by the difference between Cr(VI) reduced and Cr(III) in solution (Figure 1B). It should be noted that even in the Hepes case, Cr(III) in solution significantly exceeds the solubility of Cr(OH)₃(s) (8). Therefore, Hepes also complexes Cr(III), but to a considerably lesser extent than Tris. Similar to the Tris case, the amount of Cr(VI) reduced plateaus after about 24 h but the amount of Cr(VI) reduced per cell is half of that with Tris.

TEM micrographs of strain MR-4 cells grown in the presence of 30 mM Tris or Hepes illustrate the extensive deposition of Cr(OH)₃(s) both intracellularly (Figures 1B and 1C, SI) and extracellularly (Figure 1B, SI) in the case of Hepes but show no evidence of precipitation in the presence of Tris (Figure 1A, SI). Previous research (6) has shown that outer membrane cytochromes are, in part, responsible for the reduction of Cr(VI) in strain MR-1. Thus, it is reasonable to assume that the deposition of Cr(OH)₃(s) around the periphery of the cell in the Hepes case is due to the nearby reduction of Cr(VI) and subsequent precipitation of Cr(III). In contrast, the intracellular localization of Cr(OH)₃(s)

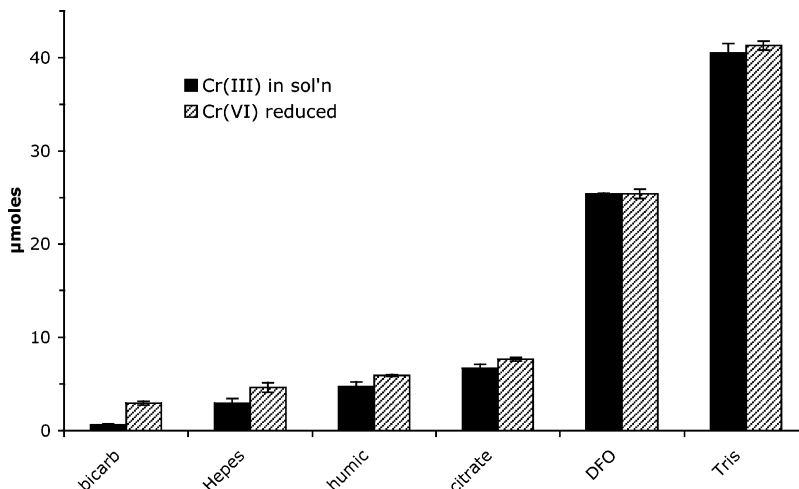


FIGURE 2. Total amount of Cr(VI) reduced by strain MR-4 in SM and the corresponding amount of solution Cr(III) after cells cease to reduce Cr(VI). Tris (30 mM), DFO (5 mM), citrate (5 mM), humic acid (1%), Hepes (30 mM), and bicarbonate (30 mM). Error bars indicate the range for duplicates.

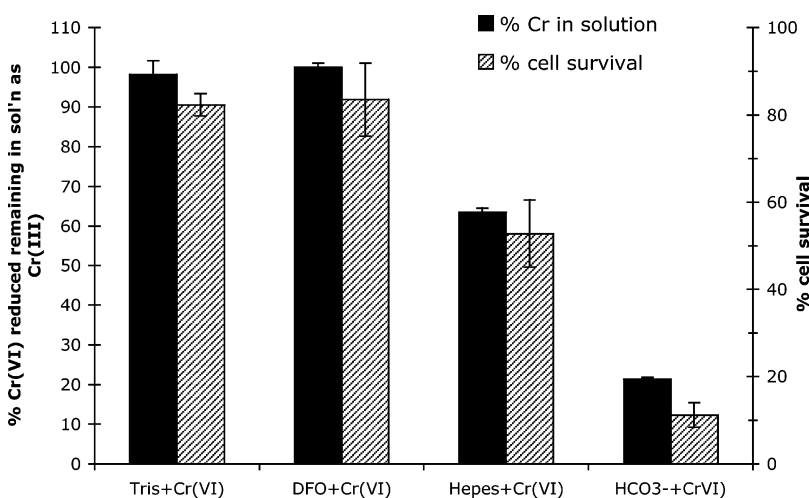


FIGURE 3. Fraction of Cr(VI) reduced that does not precipitate and that remains in solution as Cr(III) and the corresponding survival of cells after they ceased reducing Cr(VI) (relative to the initial time) in the presence of potential complexing agents in SM: Tris (30 mM), DFO (5 mM), Hepes (30 mM), bicarbonate (30 mM). Error bars indicate the range for duplicates.

suggests that the fraction of the Cr(III) produced that does not precipitate extracellularly first enters the cells then precipitates. The intracellular localization of $\text{Cr}(\text{OH})_3(\text{s})$ was also observed in strain MR-1 in the presence of bicarbonate (4). We hypothesize that it is the intracellular precipitation of Cr(III) that is responsible for the deleterious effect of Cr(III) on growth as is observed in Figure 1A.

Even though Tris and Hepes help shed light on the complex interactions linking Cr(III) complexation to Cr(VI) reduction, the environmental relevance of such a process would remain tenuous unless environmentally relevant complexing agents were found to lead to a similar phenomenon. Consequently, we considered the effect of four environmentally relevant complexing agents on Cr(VI) reduction. Desferrioxamine (DFO) is a trihydroxamate siderophore produced by bacteria and fungi and is known to bind Cr(III) effectively (9). Citrate is a plant and bacterial metabolite commonly found in soils and known to complex cations (10). Humic acids are byproducts of the biodegradation of leaves and other organic matter detritus and can be found in high concentrations in areas with decomposing plant biomass. Bicarbonate is ubiquitous in the environment, especially in limestone rich areas and is a poor complexing agent of Cr(III) (10). The concentrations of the complexing agents considered are high relative to those found in the

environment and are intended to illustrate the marked difference in complexing strength between the compounds and the concomitant effect on Cr(VI) reduction and cell survival (Figures 2 and 3).

A comparison of the amount of Cr(VI) reduced vs Cr(III) in solution for the above complexing agents is shown in Figure 2. Cr(VI) reduced represents the reduction capacity of the cells in the presence of each complexing agent and corresponds to the total amount of Cr(VI) reduced by replicate cell cultures before reduction ceases (which occurs at different times for the different complexing agents). The results clearly show that the greater the amount of Cr(III) retained in solution, the greater the total amount of Cr(VI) reduced. This result indicates that the retention of Cr(III) in solution as a complexed species after Cr(VI) reduction is crucial to maintaining active Cr(VI) reduction by strain MR-4. In other words, the formation of uncomplexed Cr(III), which will eventually precipitate as $\text{Cr}(\text{OH})_3(\text{s})$, inhibits the reduction of Cr(VI) by strain MR-4. These data support the hypothesis formulated above that the presence of Cr(III) inside the cell may be the cause of the observed inhibition: the production of Cr(III) by Cr(VI) reduction in the absence of an appropriate complexing agent leads to the availability of transient, soluble, uncomplexed Cr(III), which is either formed in or diffuses into the cytoplasm, inhibits Cr(VI) reduction and eventually

either precipitates or binds DNA and proteins (Figure 1A, SD). Previous studies illustrate the deleterious effect of Cr(III) inside eukaryotic cells: Cr(III) is reported to bind DNA (11–13), to cross-link DNA and proteins (12), to interfere with DNA replication and to increase the rate of spontaneous mutations (13).

Survival of Cells Exposed to Cr(VI) or Cr(III). The inhibition of Cr(VI) reduction activity by strain MR-4 shown above to be correlated to the formation of uncomplexed Cr(III) may be the result of a general inhibitory effect of Cr(III) on the cells. We tested that possibility by considering the correlation between reduced Cr(VI) (i.e., Cr(III)) remaining in solution and cell survival. We assessed the survival of strain MR-4 cells in the presence of complexing agents at the initiation of the experiment and after the cessation of Cr(VI) reduction which, as before, occurs at different times for the different compounds. The potential complexing agents considered were those that correspond to the largest differences in Figure 2 (i.e., Tris/DFO vs HEPES/bicarbonate). The results (Figure 3) show that there is a large difference in the survival of cells that reduced Cr(VI) in the presence of Tris or DFO as opposed to HEPES or bicarbonate. More importantly, there is a positive correlation between cell survival and the fraction of Cr(VI) reduced that remains in solution (Figure 3). For instance, in the presence of Tris or DFO, 98 or 100% of reduced Cr(VI) remains in solution, respectively. In both cases, >90% of the cells are viable as compared to the no chromium control. In contrast, in the presence of bicarbonate, 21% of Cr(VI) reduced remains in solution and about 10% of the cells were viable. The trend observed shows that the complexing capacity of the medium is directly correlated to the survival of cells after Cr(VI) reduction. The greatest proportion of viable cells is found in the samples with the least Cr(III) precipitation. However, precipitation in itself (or at least extra-cellular precipitation) may not be the cause of the inhibition. We hypothesize that the mechanism of inhibition is linked to the transient production of uncomplexed Cr(III) which enters (or is formed in) the cytoplasm.

Some of the complexing agents considered in this study, such as citrate and the siderophore DFO, are potentially relevant in the environment and could be considered in actual remediation projects to enhance the reduction of Cr(VI) and cell survival. In addition, Puzon et al. (14) recently showed that cellular organics also have the ability to complex Cr(III), which may compound the effect of the other, exogenous, complexing agents. The work by Puzon et al. (14) contributes to the complex picture of Cr-bacteria interactions and supports the central theme of this study which relies on extensive Cr(III) complexation by a host of organic compounds. The cellular localization of these compounds relative to the site of reduction of Cr(VI) will be critical in determining whether the Cr(III) complexes are formed and retained in solution or whether Cr(III) precipitates.

To test the hypothesis that the mechanism of inhibition is linked to the transient production of uncomplexed Cr(III), we conducted survival experiments similar to those shown in Figure 3 but instead of amending the media with Cr(VI) and complexing agents, we used different forms of Cr(III): Cr(III) complexed with Tris or DFO, Cr(OH)₃(s) formed prior to contact with cells or Cr(OH)₃(s) freshly precipitated in the presence of cells. The results show that complexed Cr(III) as well as aged Cr(III) precipitates do not have a significant effect on the survival of bacteria but that freshly precipitated Cr(OH)₃(s) does considerably impact cell survival (Figure 4A). The number of CFU obtained over time for either Tris, DFO (data not shown) or Cr(OH)₃(s) are similar to the no Cr control (Figure 4A). In the case of Tris, there is a slight inhibitory effect of Cr(III) as evidenced by less growth at 6 and 9 h (Figure 4A). For Cr(OH)₃(s), the CFU profile is almost identical

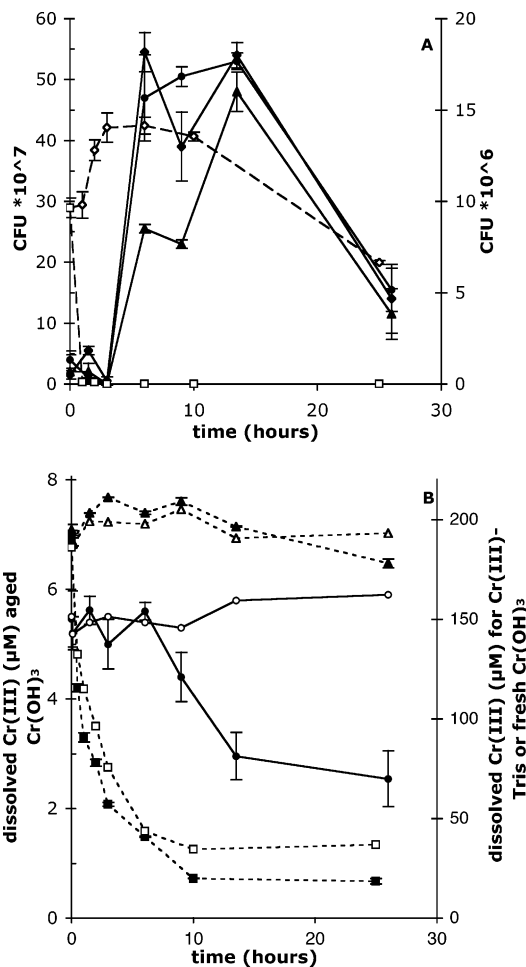


FIGURE 4. Effect of Cr(III) species on cell survival. **A.** Cell survival (in $CFU \times 10^7$) in the presence of 0.2 mM Cr(III)-Tris complex (▲), 50 mM aged Cr(OH)₃(s) (●) or in the absence of Cr(III) (◆) in SM. Similarly, cell survival (in $CFU \times 10^6$) in MSM in the presence of 5 mM freshly precipitated Cr(OH)₃(s) (□) or in the absence of Cr(III) (◇). **B.** Dissolved Cr(III) with 0.2 mM Cr(III)-Tris in the presence (▲) and absence (△) of cells, with 50 mM aged Cr(OH)₃(s) in the presence (●) and absence (○) of cells or with 5 mM freshly precipitated Cr(OH)₃(s) in the presence (■) and absence (□) of cells. For both graphs, the dotted lines correspond to the right y-axis and the solid lines to the left y-axis. Error bars indicate the range for duplicates.

to the no Cr control (Figure 4A). In contrast, the CFU profile for freshly precipitated Cr(OH)₃(s) shows that there is a significant decrease in the survival of cells. In the case of freshly precipitated Cr(OH)₃(s), the total initial CFU is different than for Cr(III)-Tris or aged Cr(OH)₃(s) because of the need to use a different medium to minimize the complexation of Cr(III). Strain MR-4 does not grow as well on MSM medium as on SM (Figure 4A) probably due to the fact that galactose is not as efficient an electron donor as lactate and the pH is 6 instead of 8. But comparing the CFU in the presence of fresh Cr(OH)₃(s) and in the absence of Cr in the same medium (Figure 4A), it is clear that fresh Cr(OH)₃(s) leads to a precipitous decrease in the survival of strain MR-4.

The corresponding profiles of dissolved Cr(III) in the Cr(III)-Tris experiments do not vary significantly over the course of the experiment in the presence or absence of cells (Figure 4B). For aged Cr(OH)₃(s), the solution concentration of Cr is initially $\sim 5 \mu M$ both in the presence and absence of cells. It does not change over time in the absence of cells but decreases over time in the presence of cells (Figure 4B). This probably indicates that Cr(III) sorbs onto cells or that they

serve as nucleation centers and promote further precipitation. The presence of measurable amounts of soluble Cr(III) in equilibrium with the aged solid phase may be on account of complexation with lactate (15). These results are consistent with those obtained in the Cr(VI) system: in the presence of Tris, Cr(III) is complexed as it is produced through Cr(VI) reduction and cell survival and activity is maintained (Figures 1 and 3). In the case of freshly precipitated Cr(OH)₃(s), the behavior is more complex. In a well-buffered system, as the one we are using, the addition of Cr(III) as an acidic solution of CrCl₃ results initially in the presence of Cr³⁺ and other soluble species in solution, followed by the precipitation of Cr(OH)₃(s) without a significant change in pH. The initial concentration of CrCl₃ added (Figure 4B) was 5 mM. However, the concentration dropped within minutes (the time required to sample) to 190 μM due to rapid precipitation of Cr(OH)₃(s). Subsequently, the precipitation is relatively slow as evidenced by the gradual decrease in solution Cr(III) shown in Figure 4B. In this experiment, we used 20 nm filters instead of the 0.22 μm filters previously used in an effort to minimize the effect of colloidal Cr(OH)₃(s).

As seen in Figure 4A, the addition of CrCl₃ to cells causes an abrupt drop in cell survival at the highest total Cr concentration considered (5 mM). At lower concentrations, a similar effect is observed (data not shown) at 50 and 100 μM of total Cr(III) added as CrCl₃, 35 and 55 μM of Cr are still in solution after 3 h, and only 65% and 51% of the cells are viable, respectively.

The main difference between preformed Cr(OH)₃(s) (which has little effect on cells) and Cr(OH)₃(s) formed in the presence of cells (which leads to loss of survival) is that in the latter case, Cr(III) is available to enter the cells in significant quantities. We conclude that transiently available Cr(III), possibly Cr³⁺, is the cause of the decrease in cell survival observed in Figure 4B and may also be the cause of the decrease in cell survival when Cr(VI) is reduced by strain MR-4 in the absence of an adequate Cr(III) complexing agent. Again, this is consistent with the results in the Cr(VI) system: in the absence of a complexing agent, Cr(III) produced through Cr(VI) reduction may be transiently stable and cause the decrease in cell survival and activity observed (Figures 1 and 3). The effect of Cr on strain MR-4 is then ultimately due to the slow kinetics of precipitation of a fraction of the Cr(III) produced (or added).

Effect of Cr(VI) and Cr(III) on Gene Expression. We examined the effect of Cr species on the expression of four genes encoding proteins involved in DNA replication: three subunits of DNA polymerase III (*dnaE*, *dnaN*, and *holA* encoding the alpha, beta, and delta subunits respectively) as well as DNA polymerase I encoded by *polA*. DNA polymerase III is the primary enzyme involved in DNA replication whereas DNA polymerase I fills in the gaps resulting from the removal of RNA primers. Out of the four genes investigated, three show obvious downregulation in the presence of soluble, uncomplexed Cr(III): *dnaN*, *holA*, *polA*, are downregulated between 15 and 22 fold (Table 2, SI). *dnaE* is only downregulated 1.3 fold (Table 2-SI). These results suggest that Cr(III) interferes with transcription and/or DNA replication possibly through binding DNA, protein or both and indicate a deleterious effect on the basic cell machinery.

In addition, the effect of Tris on the expression of *polA* was investigated. In the presence of Tris as a Cr(III)-Tris complex, the expression of *polA* does not change significantly (1.3 fold) relative to the no chromium control. However, the presence of Tris during the reduction of Cr(VI), leads to the significant (9 fold) upregulation of *polA* relative to the absence of chromium (Table 2, SI). Upregulation or the same level of expression relative to no Cr indicates that the cells are actively transcribing DNA at the same or greater level than in the absence of Cr. Thus, Tris alleviates the toxic effect of

chromium whether as Cr(III) or during Cr(VI) reduction because the expression of *polA* is significantly greater when Tris is present as when it is absent. In contrast, in the case of Cr(VI) and Hepes, *polA* was downregulated indicating a deleterious effect of Cr(VI) reduction in the absence of an appropriate complexing agent. This result is consistent with the effect of complexing agents on cell activity and survival (Figures 1 and 3) and points to Cr(III) as a toxic form of chromium.

Based on the results of this study we hypothesize that the mechanism of toxicity of chromium to *Shewanella* sp. MR-4 is due to Cr(III) either as the chromium cation (Cr³⁺) or a cationic hydroxyl complex of the metal (such as Cr(OH)²⁺ or Cr(OH)₂⁺) present in the cytoplasm which binds nonspecifically to DNA and other cellular components and inhibits transcription and, possibly, DNA replication. It is also apparent from this study that Cr(VI) as the chromate ion is not, in itself, a toxic species because it has no effect on cell growth prior to the onset of reduction. Chromium either enters the cell as chromate through assimilatory sulfate uptake channels, as is the case for *Pseudomonas fluorescens* (16), and is reduced in the cytoplasm, or enters as a cation (one of the species shown above) after the reduction has taken place on the outside of the outer membrane. If there is a complexing agent present in sufficiently high concentration in the medium to complex Cr(III), the complexing agent binds Cr(III) and prevents its damaging effect. If Cr(VI) enters the cell and is reduced intracellularly, it is unclear whether the complexing agent needs to enter the cytoplasm (Tris is known to enter cells (17) or whether the Cr(III) formed in the cytoplasm is efficiently pumped out of the cell. It is clear, however, that rendering soluble Cr(III) unavailable through complexation or as an aged Cr(OH)₃(s) precipitate prevents the inhibitory effect observed in the presence of uncomplexed Cr(III) and allows sustained Cr(VI) reduction.

Our result is comparable to that obtained for the uranyl cation (UO₂²⁺). Uncomplexed uranium (UO₂²⁺) or uranium in the form of a hydroxyl complex, binds to the membrane of *Pseudomonas fluorescens* cells and inhibits metabolism through an unknown mechanism (18). However, UO₂²⁺ complexed to carbonate, does not inhibit cell metabolism (18). The same behavior was observed for *Shewanella oneidensis* MR-1 (Yi Wen, unpublished data). The main differences between the effect of U(VI) and that of Cr(III) are that (a) the inhibition is reversible for U(VI) but not for Cr(III) and (b) there is no evidence of the cation entering the cytoplasm in the case of U(VI) whereas there is in the case of Cr(III).

Complexation of Cr(III) by organic compounds is well-known (14, 19). However, this is the first time, to our knowledge, that microbial Cr(VI) reduction and cell survival are enhanced by the complexation of the product of the reduction (i.e., Cr(III)). Unfortunately, complexation also retains Cr(III) in solution and does not immobilize chromium as a precipitate which is desirable for remediation purposes. The implication is that in order to attain effective bioremediation by stimulating an organism such as *Shewanella* spp., known to be present in at least some of the contaminated sites (2), the toxicity of Cr(III) during Cr(VI) reduction to cells must be overcome, possibly through initial complexation followed by precipitation. It is likely that in soil or sediment environments, the Cr(III) complex formed is not as long-lasting as it is under sterile laboratory conditions due to the depletion of the complexing agent through biological degradation. If the complexing agent is biodegraded, Cr(III) would eventually precipitate as Cr(OH)₃(s) and lead to the desired immobilization of Cr. From a practical standpoint, it may be advantageous to amend the site with a complexing agent that efficiently binds Cr(III) because doing so may stimulate the reduction of Cr(VI) and cell survival but

attention must be paid to the persistence of Cr(III) as a soluble complex.

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Supporting Information Available

Additional tables showing the primers used for RT-PCR and qPCR and fold change expression, and a TEM micrograph showing the strain MR-4 cells. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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