Effects of Chromium(VI) and Chromium(III) on *Desulfovibrio vulgaris* Cells

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Abstract

Desulfovibrio vulgaris ATCC 29579 is a well studied sulfate reducer that has known capabilities of reducing heavy metals and radionuclides, like chromium and uranium. Cultures grown in a defined medium (i.e. LS4D) had a lag period of approximately 40 h when exposed to 50 µM of Cr(VI). Substrate analysis revealed that although chromium is reduced within the first 5 h, growth does not resume for another 35 h. During this time, small amounts of lactate are still utilized but the reduction of sulfate does not occur. Sulfate reduction occurs concurrently with the accumulation of acetate approximately 40 h after inoculation, when growth resumes. Similar amounts of hydrogen are produced during this time compared to hydrogen production by cells not exposed to Cr(VI); therefore an accumulation of hydrogen cannot account for the utilization of lactate. There is a significant decrease in the carbohydrate to protein ratio at approximately 25 h, and this result indicated that lactate is not converted to glycogen. Most probable number analysis indicated that cell viability decreased steadily after inoculation and reached approximately 6 x 10^4 cells/ml 20 h post-chromium exposure. Regeneration of reducing conditions during chromium exposure does not induce growth and in fact may make the growth conditions even more unfavorable. This result suggested that an increase in E_h was not solely responsible for the decline in viability. Cell pellets collected 10 h after chromium-exposure were unable to resume growth when suspended into fresh medium. Supernatants from these pellets were able to support cell growth upon re-inoculation. *D. vulgaris* cells treated with a non-dose dependent addition of ascorbate at the same time of Cr(VI) addition did not enter a lag period. Ascorbate added 3 h post-Cr(VI) exposure did not prevent the growth lag. These results indicated that *Desulfovibrio* utilized lactate to reduce Cr(VI) without the reduction of sulfate, that the decline in cell viability and cell growth was most likely a consequence of Cr(III), and that an organic ligand could protect *D. vulgaris* cells from Cr(III) toxicity. Lactate consumption decoupled from sulfate reduction in the presence of Cr(VI) could provide organic carbon for organo-Cr(III) complexes.

Introduction

Chromium contamination is a common contaminant of both soil and water and is considered both carcinogenic and mutagenic. Cr (VI) is a soluble form that can easily pass through cell membranes, oxidizing into reactive speciations (Cr (V) & Cr (IV)) and generating free radicals that can damage DNA. Reduction to Cr (III) renders this metal less soluble and less toxic and is an advantageous speciation to maintain within contaminated soil and water. Many microorganisms have demonstrated the ability to reduce (VI). Of particular interest is the metal reduction capabilities of SRBs. *Desulfovibrio vulgaris* is the model SRB and has been shown to reduce metals, metalloids, and radionuclides. This cell-mediated reduction involves hydrogenases and cytochrome *c3* as well as the hydrogen sulfide generated (Lovely et al., 1994; Chardin et al., 2002). Our previous results show a decoupling of lactate oxidation from sulfate reduction during a period of growth inhibition and Chardin et al. (2002) demonstrated energy production in the absence of growth during Cr (VI) exposure. Decoupling of lactate oxidation with sulfate reduction has also been documented for *D. vulgaris* in the presence of U (VI) and Fe (III) (Elias et al., 2004).

Complexes formed with Cr (III) are currently of interest and may explain how organisms like *D. vulgaris* can survive Cr stress. Previous studies have shown that Cr(III)-NAD+ could be formed as well as Cr (III) complexes with ascorbate, serine, malate, oxaloacetate, and glutathione, just to name a few (Puzon, et al. 2002 & 2005). Mabbett et al. demonstrated that resting *D. vulgaris* cells could reduce Cr (VI) faster in the presence of ligands, such as citrate, diethylenetriamine pentaacetic acid (DPTA), and ethylenediamine tetraacetic acid (EDTA). Conversely, Goulhen et al. (2006) hypothesize that a trivalent chromium phosphate precipitates on the cell membrane and in the periplasm, rendering the cell unable to take up nutrients, therefore killing the cell. This in turn generates a subpopulation of cells that has not been mineralized to begin to divide when conditions are more favorable.
These results, taken together, indicate two subsets of cells may exists; one which reduces the Cr (VI) and the other to produce a organic ligand to protect the remaining unmineralized cells.

RESULTS

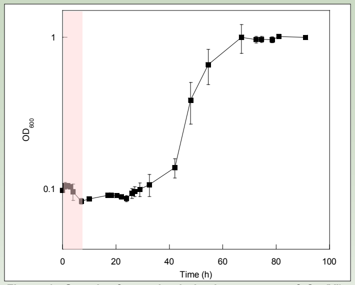

Figure 1: Growth of *D. vulgaris* in the presence of Cr (VI). Cultures were started at a cell density of 0.06 OD_{600} in LS4D amended with 50 µM Cr(VI). Growth did not ensue for 40 h post-inoculation. Pink shading indicates the time it takes for *D. vulgaris* to reduce the Cr(VI) to Cr(III).

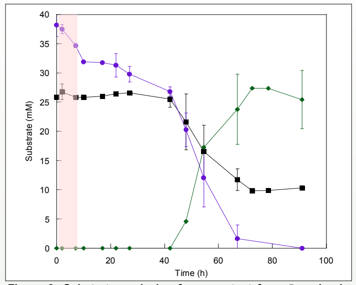

Figure 2: Substrate analysis of supernatant from *D. vulgaris* cultures inoculated in LS4D in the presence of 50µM Cr(VI). Sulfate levels remain constant until growth begins 40 h after inoculation. Acetate does not begin to accumulate until growth was observed and sulfate is reduced. Lactate levels declined during the reduction of Cr(VI) to Cr (III) even though sulfate is not utilized. Pink shading indicates time of Cr(VI) reduction to Cr(III).

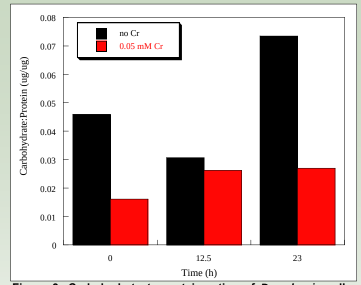

Figure 3: Carbohydrate to protein ratios of *D. vulgaris* cells exposed to Cr (VI). Red bars indicate ratios of cells exposed to Cr(VI) while Black bars represent the control. Carbohydrate production increases slightly in cells exposed to Cr(VI) after 12 h, but remains constant during subsequent time points. Control samples indicated an increase in carbohydrate production approximately one day after inoculation and is almost 3 fold higher compared to cells exposed to Cr(VI). These results indicate that lactate utilization during Cr(VI) reduction does not go towards generation of glycogen.

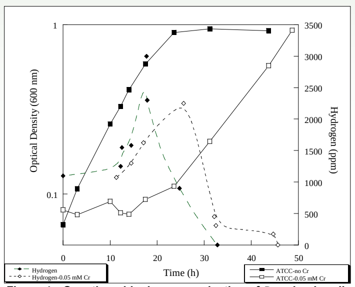

Figure 4: Growth and hydrogen production of *D. vulgaris* cells exposed to Cr(VI). Data shows that cells produced similar amounts of hydrogen whether or not they were exposed to chromium.
——— represent growth
- - - - represent hydrogen levels
■, ♦ represent cells with no Cr (VI)
□, ◊ represent cells grown in the presence of Cr (VI)

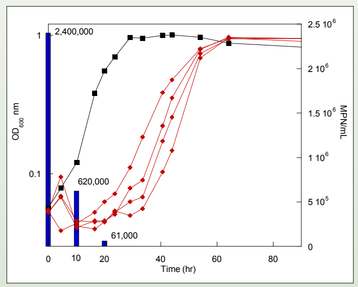

Figure 5: Growth and viability of cells exposed to 50 µM Cr (VI). Results show a decrease in cell viability after chromium exposure with only 6 x 10^4 cells present 20 h after exposure.
——— represents growth of cells with no Cr (VI)
——— represents growth of cells exposed to Cr (VI)
▌ represents cells viability

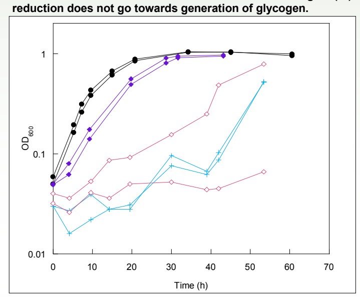

Figure 6: Supernatants of *D. vulgaris* cultures were collected via filtration 10 h and 20 h post-exposure to Cr(VI) to determine if an extracellular inhibitor was affecting cell growth. Results indicated that the supernatant did not inhibit cell growth when inoculated with logarithmic growing cells. Cells pellets that were exposed to Cr(VI) did not resume growth when suspended into fresh LS4D medium. These results indicated that individual cells could not be immediately resuscitated and/or a by-product of Cr(VI) reduction was associated with the cells.
——— 10 h supernatant used to suspend cells
——— 20 h supernatant used to suspend cells
——— 10 h pellet suspended in LS4D
——— 10 h pellet suspended in LS4D plus titanium citrate

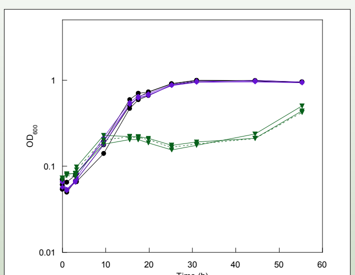

Figure 7: Regeneration of reducing conditions in order to determine if increase in E_h was contributing to growth inhibition. Sulfide addition may have initially resuscitated growth, but cells began to lag at 10 h and growth was retarded up to 50 h post-inoculation.
——— LS4D
——— LS4D plus 1.6 mM sodium sulfide
——— LS4D plus 1.6 mM sodium sulfide and 50 µM Cr (VI)

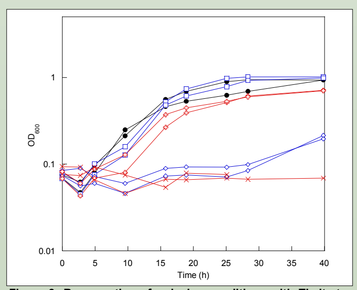

Figure 8: Regeneration of reducing conditions with Ti-citrate and cysteine to determine if E_h was contributing to growth inhibition. Growth continued to lag with the addition of the reducing agents and did not resume even at 40 h.
● Control
□ LS4D plus TiCl
◊ LS4D plus TiCl and 50µM Cr (VI)
+ LS4D plus cysteine
X LS4D plus cysteine and 50µM Cr (VI)

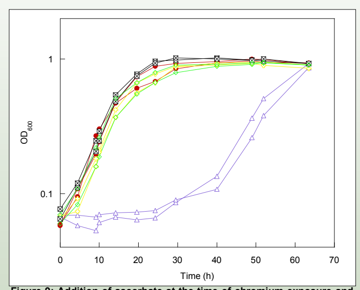

Figure 9: Addition of ascorbate at the time of chromium exposure and 5 h post exposure. Results show that the addition of ascorbate at the time of exposure prevented arrested cell growth and cells had a generation time similar to the control. Addition of ascorbate 5 h post exposure did not rescue cell growth and the lag continued for 40 h.
□ LS4D (control)
◊ LS4D plus 50mM ascorbate
● LS4D plus 48h Cr(III)-ascorbate complex
● LS4D plus 50 µM Cr (VI) and 50mM ascorbate
Δ LS4D plus 50 µM Cr (VI) and 50mM ascorbate after 3h

METHODS

Growth: *D. vulgaris* ATCC 29579 was growth in the minimal medium LS4D. Pre-cultures were grown until an OD600 of 0.5-0.7 was reached. Cells were harvested by centrifugation at RT, for 10 min in under anaerobic conditions. The pellets were washed twice, suspended in fresh LS4D medium and used immediately for inoculation. Cr (VI) (50µM) was added into the medium at the time of inoculation where appropriate.

Substrate analysis: Lactate, acetate, and sulfate levels were measure on a Metrohm IC with an Metrosep organic acid and Metrosep anion Supp 5 column, respectfully.

Protein & Carbohydrate levels: Protein levels were measured using the Lowry colormetric assay while carbhydrates were measured using the cysteine-sulfuric acid colormetric assay.

Viability: Cell viability was determined using the Most Probable Number (MPN) method. Serial dilutions were generated at each time point in sets of 3. After 3 days, tubes were observed for growth and scored with either a (+) or (-). Results were entered into an MPN calculator and cell numbers were determined.

Filtration Experiment: Cultures were grown as described above. At 10 h and 20 h cultures were pelleted, the supernatant was removed and filtered through a 0.2µm filter into a sterile, N2 flushed tube. Supernatants were then inoculated with freshly washed cells. Pellets were also collected at 10 h and suspended in fresh medium containing no chromium. One set was reduced with titanium citrate before suspension.

Regeneration of Reducing Conditions: LS4D was reduced before inoculation with the following: 1.6mM sodium sulfide, titanium citrate, and cysteine. Reduced medium was inoculated with washed cells and Cr (VI) was added to one set of tubes.

Ascorbate-Cr(III) complex: 50mM of ascorbate was added to LS4D inculated with washed cells and 50µM Cr (VI) at the time of inoculation and 3 h post exposure. Control tubes containing no ascorbate, 50mM ascorbate, and a 48 h ascorbate-Cr(III) complex were also monitored.

DISCUSSION

- 50 µM Cr (VI) inhibits growth of *D. vulgaris* cells for 40 h
- Substrate analysis revealed that lactate is being utilized during this static state but sulfate is not being reduced and acetate did not accumulate
- Hydrogen and internal carbohydrate production do not increase during this lag phase, and these results suggested that lactate was being utilized for other means besides carbon reserves and/or reducing equivalents
- Viable cells decreased in numbers during this lag phase which may indicate Cr (III) precipitates may be generated and deposited on the cell surface
- Cells exposed to Cr (VI) were unable to grow in the presence of fresh medium, corresponding with the cell viability results
- Cells exposed to Cr (VI) need time to deal with oxidative stresses generated during Cr (VI) reduction or may be inhibited by the presence of Cr (III)
- Regeneration of reducing condition does not prevent growth inhibition and may even provide a more toxic environment for the cells
- The organic compound, ascorbate, was able to rescue cells from the deleterious effects of Cr (VI)/Cr (III) indicating that an organo-Cr (III) compound could prevent growth inhibition
- *D. vulgaris* cells may be generating an organic compound to bind with the Cr(III) produced during the 40 h lag period

Acknowledgements

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