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

M.E. Clark[1,2], A. Klonowska[1], S.B. Thieman[1], B. Giles[3], J.D. Wall[3∫], and M.W. Fields[2,4∫]

[1]Miami University, Oxford, OH; [2]Center for Biofilm Engineering, Montana State University, Bozeman, MT; [3]University of Missouri, Columbia, MO;
[4]Department of Microbiology, Montana State University; [∫]Virtual Institute of Microbial Stress and Survival

MONTANA STATE UNIVERSITY

VIMSS Virtual Institute for Microbial Stress and Survival

a National Science Foundation Engineering Research Center in the MSU College of Engineering

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

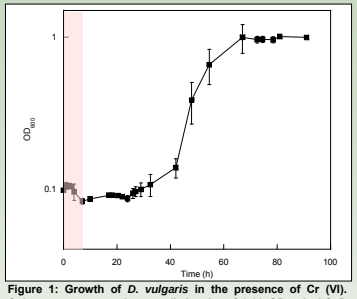

Figure 1: Growth of *D. vulgaris* in the presence of Cr (VI). Cultures were started at a cell density of 0.06 OD_{600} in LS4D amended with 50 µM Cr(VI). Growth did not ensue for 40 h post-inoculation. Pink shading indicates the time it takes for *D. vulgaris* to reduce the Cr(VI) to Cr(III).

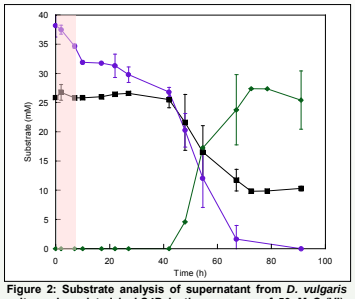

Figure 2: Substrate analysis of supernatant from *D. vulgaris* cultures inoculated in LS4D in the presence of 50µM Cr(VI). Sulfate levels remain constant until growth begins 40 h after inoculation. Acetate does not begin to accumulate until growth was observed and sulfate is reduced. Lactate levels declined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

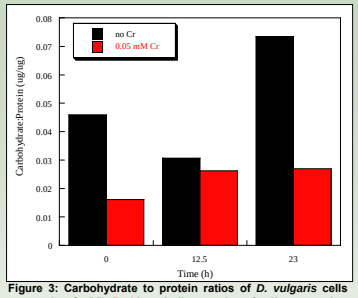

Figure 3: Carbohydrate to protein ratios of *D. vulgaris* cells exposed to Cr (VI). Red bars indicate ratios of cells exposed to Cr(VI) while Black bars represent the control. Carbohydrate production increases slightly in cells exposed to Cr(VI) after 12 h, but remains constant during subsequent time 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.

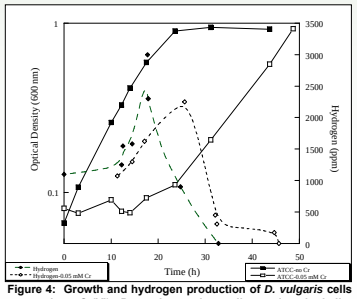

Figure 4: Growth and hydrogen production of *D. vulgaris* cells exposed to Cr(VI). Data shows that cells produced similar amounts of hydrogen whether or not they were exposed to chromium.
——— represent growth
- - - - represent hydrogen levels
■, ♦ represent cells with no Cr (VI)
□, ◊ represent cells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

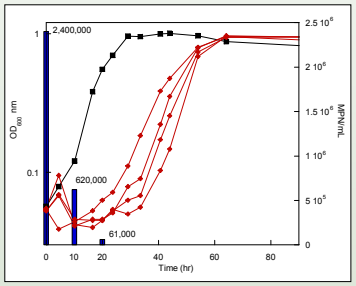

Figure 5: Growth and viability of cells exposed to 50 µM Cr (VI). Results show a decrease in cell viability after chromium exposure with only 6 x 10^4 cells present 20 h after exposure.
——— represents growth of cells with no Cr (VI)
——— represents growth of cells exposed to Cr (VI)
▮ represents cells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

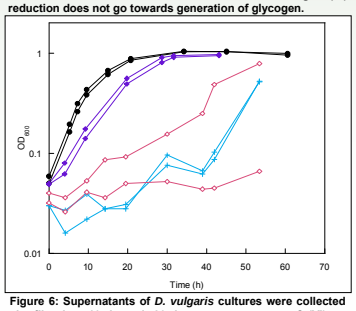

Figure 6: Supernatants of *D. vulgaris* cultures were collected via filtration 10 h and 20 h post-exposure to Cr(VI) to determine if an extracellular inhibitor was affecting cell growth. Results indicated that the supernatant did not inhibit cell growth when inoculated with logarithmic growing cells. 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

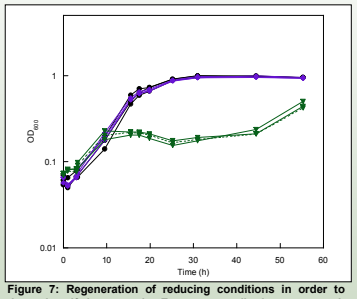

Figure 7: Regeneration of reducing conditions in order to determine if increase in E_h was contributing to growth inhibition. Sulfide addition may have initially resuscitated growth, but cells began to lag at 10 h and growth was retarded up to 50 h post-inoculation.
——— LS4D
——— LS4D plus 1.6 mM sodium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

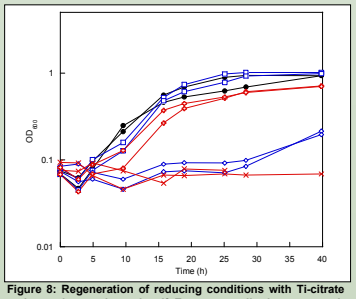

Figure 8: Regeneration of reducing conditions with Ti-citrate and cysteine to determine if E_h was contributing to growth inhibition. Growth continued to lag with the addition of the reducing agents and did not resume even at 40 h.
● Control
□ LS4D plus TiCl
◊ LS4D plus TiCl and 50µM Cr (VI)
+ LS4D plus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

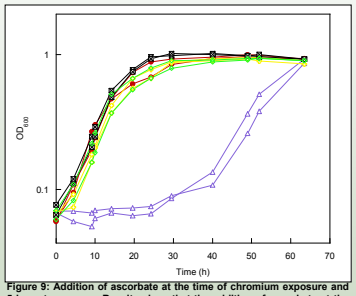

Figure 9: Addition of ascorbate at the time of chromium exposure and 5 h post exposure. Results show that the addition of ascorbate at the time of exposure prevented arrested cell growth and cells had a generation time similar to the control. Addition of ascorbate 5 h post exposure did not rescue cell 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
△ LS4D plus 50 µM Cr (VI) and 50mM ascorbate after 3h

METHO

Growth: *D. vulgaris* ATCC 29 minimal medium LS4D. Pre-cultu OD600 of 0.5-0.7 was reached. centrifugation at RT, for 10 m conditions. The pellets were wash fresh LS4D medium and used imm Cr (VI) (50µM) was added into th inoculation where appropriate.

Substrate analysis: Lactate, a were measure on a Metrohm IC w acid and Metrosep anion Supp 5 c

Protein & Carbohydrate lev measured using the Lowry c carbhydrates were measured usi acid colormetric assay.

Viability: Cell viability was det Probable Number (MPN) metho generated at each time point in set were observed for growth and scor). Results were entered into an numbers were determined.

Filtration Experiment: Cul described above. At 10 h and 20 the supernatant was removed and filter into a sterile, N2 flushed then inoculated with freshly washe collected at 10 h and suspen containing no chromium. One titanium citrate before suspension.

Regeneration of Reducing reduced before inoculation with sodium sulfide, titanium citrate, medium was inoculated with wash added to one set of tubes.

Ascorbate-Cr(III) complex: added to LS4D inculated with wa (VI) at the time of inoculation Control tubes containing no asc and a 48 h ascorbate-Cr(III) compl

DISCUSS

➢ 50 µM Cr (VI) inhibits growth of h

➢ Substrate analysis revealed that during this static state but sulfate i acetate did not accumulate

➢ Hydrogen and internal carbohyd increase during this lag phase, and that lactate was being utilized for c carbon reserves and/or reducing e

➢ Viable cells decreased in number which may indicate Cr (III) precipi and deposited on the cell surface

➢ Cells exposed to Cr (VI) were un presence of fresh medium, corresp viability results

➢Cells exposed to Cr (VI) need tim stresses generated during Cr (VI) r inhibited by the presence of Cr (III

➢ Regeneration of reducing condit growth inhibition and may even pr environment for the cells

➢ The organic compound, ascorbat cells from the deleterious effects of indicating that an organo-Cr (III) growth inhibition

➢ *D. vulgaris* cells may be generat to bind with the Cr(III) produced d

Acknowledg

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