



Carbon-dependent chromate toxicity mechanism in an environmental *Arthrobacter* isolate

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ABSTRACT

Arthrobacter spp. are widespread in soil systems and well-known for their Cr(VI) reduction capabilities making them attractive candidates for *in situ* bioremediation efforts. Cellulose drives carbon flow in soil systems; yet, most laboratory studies evaluate *Arthrobacter*-Cr(VI) interactions solely with nutrient-rich media or glucose. This study aims to determine how various cellulose degradation products and biostimulation substrates influence Cr(VI) toxicity, reduction, and microbial growth of an environmental *Arthrobacter* sp. isolate. Laboratory culture-based studies suggest there is a carbon-dependent Cr(VI) toxicity mechanism that affects subsequent Cr(VI) reduction by strain LLW01. Strain LLW01 could only grow in the presence of, and reduce, 50 μ M Cr(VI) when glucose or lactate were provided. Compared to lactate, Cr(VI) was at least 30-fold and 10-fold more toxic when ethanol or butyrate was the sole carbon source, respectively. The addition of sulfate mitigated toxicity somewhat, but had no effect on the extent of Cr(VI) reduction. Cell viability studies indicated that a small fraction of cells were viable after 8 days suggesting cell growth and subsequent Cr(VI) reduction may resume. These results suggest when designing bioremediation strategies with *Arthrobacter* spp. such as strain LLW01, carbon sources such as glucose and lactate should be considered over ethanol and butyrate.

1. Introduction

Chromate (Cr(VI)) is a widespread heavy metal contaminant in soil systems [1–7]. It is a known human carcinogen by the US Toxicological Program and the International Agency for Research on Cancer with the US EPA reference dose of 5 μ g/kg/d based on a one year study in rats [8]. The reduction of Cr(VI) to the less water soluble, less toxic trivalent form (Cr(III)) is therefore advantageous *in situ*. The microbial reduction of Cr(VI) to Cr(III) is one potential mechanism to remediate contaminated soil systems; however, microbes must be capable of surviving in the presence of this contaminant to contribute to its remediation.

Arthrobacter spp. are ubiquitous in soil systems [9,10] and are well known for their high tolerance to a variety of toxic compounds including Cr(VI). *Arthrobacter* spp. mitigate toxicity through the use of cellular efflux pumps [5,11], biosorption to extracellular polymeric substances produced by the cells [12,13], through the direct reduction of Cr(VI) to its less toxic form Cr(III) [8,14,15], and through the indirect reduction of Cr(VI) to Cr(III) due to aerobic microbial metabolism [16]. These characteristics make *Arthrobacter* spp. attractive candidates for a wide variety of environmental applications including Cr(VI)

remediation efforts as biosorbents [13,17] and biostimulation targets for metal reduction [18–20]. The widespread presence of *Arthrobacter* spp. at known metal contaminated sites [3–9] and a wide variety of other soil systems [1,2] suggests that if microbial growth is stimulated to promote chromate reduction, *Arthrobacter* spp. may be significant contributors to this process.

Soil systems are complex and microbial Cr(VI) reduction *in situ* is affected by a variety of conditions including soil pH, moisture, oxygen, redox potential, organic matter, the availability of other electron donors and acceptors, and the microbial community structure [8,16,21,22]. Cellulose is a primary source of carbon in terrestrial soil systems and therefore is an important driver in carbon cycling *in situ* [23–25] and ultimately in microbial Cr(VI) reduction. It has been well-established that the carbon source available can influence both the microbial community structure and a community's ability to reduce Cr(VI) [26–30]. And yet, laboratory studies have focused largely on Cr(VI)-resistance and reduction capabilities of *Arthrobacter* spp. grown on nutrient-rich media with glucose as the primary carbon source [2,3,18,20,31,32]. As glucose is rapidly consumed by microorganisms in the environment due to its high energy yield, there are a variety of

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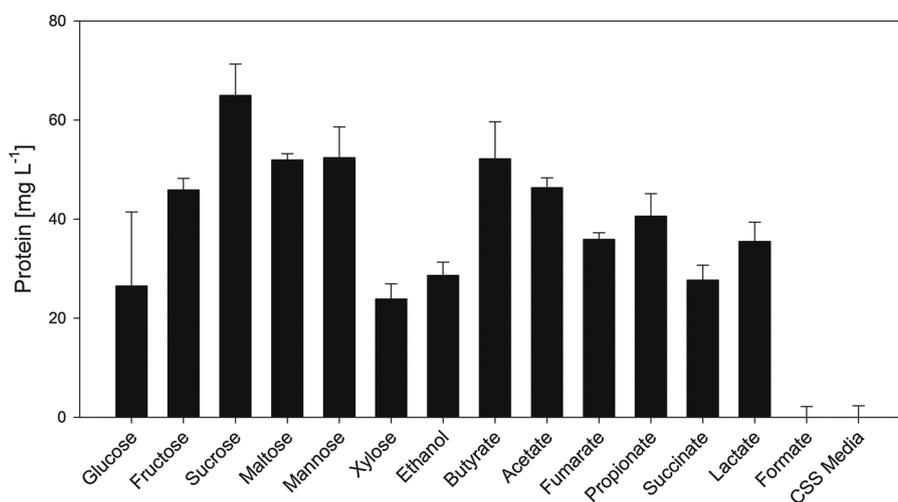


Fig. 1. Protein concentration during *Arthrobacter* sp. LLW01 growth on potential cellulose degradation products and biostimulation carbon sources after 72 h. All carbon sources were normalized to 15 mM carbon. Error bars represent \pm one standard deviation ($n = 2$).

other cellulose degradation products which would also be readily available for consumption. However, these have not been well addressed in laboratory studies. Similarly, in environments where organic carbon is added to stimulate microbial contaminant remediation [33–38] it is imperative to first understand how these organisms respond under environmentally-relevant conditions.

The Department of Energy (DOE) is responsible for a number of Cr(VI)-contaminated sites in which *Arthrobacter* spp. have been found [2,39,40]. These include Low-Level Waste (LLW) sites which contain cellulosic waste often in the form of cardboard, mop heads, and paper towels contaminated with heavy metals and radionuclides with Cr(VI) concentrations of approximately 50 μ M Cr(VI) [41,42]. This cellulosic waste is the primary source of carbon in these systems and can be degraded by microorganisms to other carbon sources including sugars, organic acids, and alcohols [43–45]. The fate of metals such as chromium in these sites is largely unknown as chromium can be released during this process enhancing transport or it can be reduced by microorganisms to a less soluble form decreasing transport. Previously, *Arthrobacter*-related 16S rRNA sequences were identified in a simulated LLW site from the Idaho National Laboratory [40] and an *Arthrobacter* sp. isolate was subsequently obtained through laboratory microcosm experiments. This study aimed to evaluate how various cellulose degradation products which may be present in LLW sites as well as other soil systems affect growth, Cr(VI) toxicity, and subsequent Cr(VI) reduction by an environmental isolate, *Arthrobacter* sp. strain LLW01. These laboratory studies address fundamental interaction between Cr(VI) and strain LLW01 when carbon metabolism varied and can be used to better predict how *Arthrobacter* spp. may contribute to Cr(VI) reduction in LLW sites and other soil systems.

2. Materials and methods

2.1. Experimental growth conditions

Arthrobacter sp. LLW01 was isolated from a soil sample collected from a simulated LLW site at the Idaho National Laboratory [40] (Supplemental Methods Isolation & Identification). Strain LLW01 was grown aerobically from frozen glycerol stocks in Tryptic Soy Broth (BD Difco, Franklin Lakes, NJ) at room temperature ($22 \pm 2^\circ\text{C}$) and shaken at 150 rpm for 20 h to reach late exponential phase. The culture was centrifuged, washed and resuspended in a defined, carbon-free medium three times. This carbon-free (CF) medium simulates the groundwater chemistry at the Idaho National Laboratory and was modified from VanEngelen et al. [46] (Table S1). Briefly, this medium contained the

following per L of medium: 80 mg NaCl, 26 mg K_2HPO_4 , 3.35 g PIPES Buffer, 0.6 mg KCl, 0.4 mg KOH, 12.7 mg Na_2SO_4 , 3.5 mg CaO, and 7.5 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ amended with 5 ml of Wolfe's vitamin solution and 1 ml SL-4 trace elements solution [47] (Table S1). The final re-suspended culture was diluted in microtiter plates with sterile medium to normalize starting cell densities to an optical density of approximately 0.2 at 600 nm (OD_{600}) (corresponding to 3.7×10^8 - 4.7×10^8 CFU mL^{-1}). Treatments were inoculated with 10% of this washed culture. Carbon source was added separately where indicated.

2.2. Batch studies

2.2.1. Carbon source utilization study

To identify which carbon sources strain LLW01 can utilize for growth, a study was performed with fourteen different substrates. The following carbon sources representing a variety of potential cellulose degradation products were used: Glucose (dextrose), fructose, maltose, mannose, sucrose, xylose, sodium acetate, sodium butyrate, sodium formate, sodium fumarate, sodium propionate, sodium succinate, sodium lactate (60% w/w lactate syrup) and ethanol (EtOH). In sterile, 24-well microtiter plates, duplicate wells were set up for each carbon source (15 mM carbon final concentration), CF medium, and strain LLW01 for a total volume of 2.5 mL per well. Carbon concentrations were chosen based on previous metal toxicity studies [46] and ensure carbon is not limiting in the experiments. Duplicate cell-free and carbon-free control wells were included. Plates were shaken at 150 rpm at room temperature ($22 \pm 2^\circ\text{C}$) and absorbance measurements were taken at 600 nm after 72 h. Protein concentrations after 72 h were determined as well. The time frame for this experiment was chosen based on preliminary growth curves with 15 mM glucose in which stationary phase had been reached.

2.2.2. Cr(VI) toxicity studies

Based on the results obtained in the 24-well plate carbon source screening study, glucose, ethanol, lactate, and butyrate were used in Cr(VI) toxicity experiments as each represents different types of potential cellulose degradation products (i.e. sugars, organic acids and alcohols). A concentration of 50 μ M Cr(VI) was used as it is close to the concentration under enrichment conditions, as well as those found in LLW sites [41,42]. These studies were carried out aerobically in 25 mL screw cap test tubes in triplicate. For each carbon source, treatments were set up containing CF medium, 15 mM carbon, and strain LLW01. Appropriate cell-free and carbon-free control treatments were also set up. Tubes were shaken at 150 rpm at room temperature ($22 \pm 2^\circ\text{C}$).

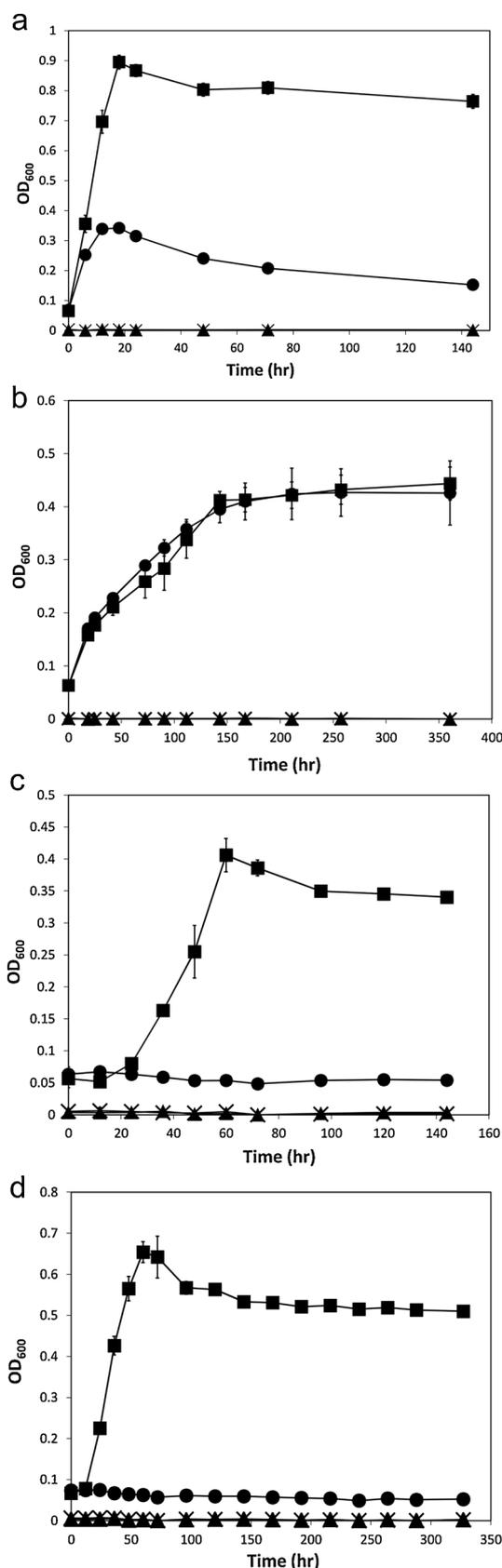


Fig. 2. Growth of *Arthrobacter* sp. LLW01 when (a) glucose, (b) lactate, (c) ethanol and (d) butyrate were the sole carbon sources in the (●) presence and (■) absence of 50 μM Cr(VI). Additional symbols: (▲) CF media only; (X) Carbon source only. Error bars represent \pm one standard deviation ($n = 3$) and are smaller than the symbols where not visible.

Absorbance measurements at 600 nm and samples for Cr(VI) analysis were taken over time.

2.2.3. Cell viability studies

Cell viability studies were conducted to determine whether Cr(VI) has a bactericidal or bacteriostatic effect on strain LLW01. For the cell viability studies treatments were scaled up to 50 mL flasks. Treatments either contained glucose or ethanol as each represent a low and high Cr(VI) toxicity carbon source, respectively. Lactate was not evaluated as no loss in cell densities, as measured by 600 nm, was observed in the presence of Cr(VI). Flasks were shaken at 150 rpm at room temperature ($22 \pm 2^\circ\text{C}$) and over time samples were taken for Cr(VI) analysis, absorbance measurements at 600 nm, and plate counts. Plate counts were conducted by serially diluting the sample in sterile phosphate buffered saline solution. Dilutions were used for drop plating in which 5 replicates of 10 μL were pipetted onto tryptic soy agar (TSA) plates for each dilution. These plates were incubated at room temperature ($22 \pm 2^\circ\text{C}$) for 24 h and colonies were counted and averaged to calculate CFU mL⁻¹.

2.2.4. Sulfate exclusion study

Cr(VI) in the form of CrO_4^{2-} has been described to enter the cell through the same transport system as sulfate (SO_4^{2-}) [48]. In an attempt to mitigate toxicity through competitively excluding Cr(VI) from the cell, SO_4^{2-} was added to the system in excess (100:1 SO_4^{2-} to CrO_4^{2-} ; 5 mM sulfate final concentration). Glucose was the provided carbon source and the same protocol as listed in the Cr(VI) toxicity batch studies was followed. Glucose was used as the sole carbon source as strain LLW01 was capable of growing in the presence of 50 μM Cr(VI), but at a reduced growth rate and maximum OD suggesting these cells were most likely to respond to mitigation methods and would have the greatest measurable response. Appropriate cell-free and carbon-free treatments were set up as well. Tubes were shaken at 150 rpm at room temperature ($22 \pm 2^\circ\text{C}$). Absorbance measurements at 600 nm and samples for Cr(VI) analysis were taken over time.

2.3. Analytical methods

2.3.1. Cr(VI) quantification

Cr(VI) concentrations were determined spectrophotometrically using the diphenylcarbazide assay as described by Nyman et al. [49]. In short, 150 μL of the sample was added to 50 μL of 0.8 N H_2SO_4 in a 96-well microtiter plate followed by the addition of 20 μL of 0.25% 1,5-diphenylcarbazide (Sigma-Aldrich, St. Louis, MO) in 100% acetone. Assay blanks were prepared by the addition of 20 μL of 100% acetone without 1,5-diphenylcarbazide. Absorbance was measured at 540 nm, adjusted with corresponding assay blanks and compared to standards made from a K_2CrO_4 stock solution (Ricca Chemical Company, Arlington, TX).

2.3.2. Protein quantification

A colorimetric protein assay, modified from Viamajala et al. [50], was used to estimate total protein. Briefly, 200 μL 1 N NaOH was added to 200 μL of sample and digested in a hot water bath at 90 °C for 10 min. After samples cooled, 28 μL of a 6:10 v/v HCl solution was added. Triplicate technical replicates of 50 μL aliquots per sample were added to a 96-well plate. Coomassie reagent (Pierce, Rockford, IL) was added to each of the wells (150 μL) and allowed to react for 15 min after which absorbance readings were taken at 595 nm. Absorbance measurements were compared to bovine serum albumin standards and technical replicate protein concentrations were averaged for each sample to provide final protein concentrations.

2.3.3. Cr(VI) reduction rates

First order reduction rates with respect to Cr(VI) concentration were estimated using the following model

Table 1
Growth Parameters of *Arthrobacter* sp. LLW01 in the Presence and Absence of 50 μM Cr(VI).

Carbon Source	Growth Rate ^a		Lag Time ^b		Estimated IC50 [μM]
	Carbon + Strain LLW01 [hr^{-1}]	Carbon + Strain LLW01 + 50 μM Cr(VI) [hr^{-1}]	Carbon + Strain LLW01 [hr]	Carbon + Strain LLW01 + 50 μM Cr(VI) [hr]	
Glucose	0.140 \pm 0.006	0.091 \pm 0.0055	0.0	0.0	48.0 \pm 1.7
Lactate	0.013 \pm 0.0005	0.013 \pm 0.0005	0.0	0.0	170.0 \pm 2.2
Butyrate	0.044 \pm 0.0021	NG	13.1 \pm 2.20	NG	9.0 \pm 1.0
Ethanol	0.032 \pm 0.0145	NG	12.0 \pm 0.02	NG	BDL

NG; No Growth Observed.

IC50; Cr(VI) concentration at which half the maximal inhibition of cell growth was observed.

BDL; Below the detection limit (5 μM Cr(VI)).

^a Error reported for at least three separate, biological replicate studies.

^b Error reported for three biological replicates within one study.

$$c\text{Cr(VI)} = c\text{Cr(VI)}_0 \cdot \exp^{-kt} \quad (1)$$

in which $c\text{Cr(VI)}_0$ is the initial Cr(VI) concentration at time zero, $c\text{Cr(VI)}$ is the Cr(VI) concentration at time (t) and k is the first order rate coefficient. Data were fitted to the model using a non-linear regression scheme by minimizing the sum of the squared residuals.

2.3.4. IC50 calculations

In this study, IC50 is defined as the Cr(VI) concentration that resulted in half the maximal cell growth as measured by absorbance at 600 nm. Strain LLW01 was grown in the presence of increasing Cr(VI) concentrations in the presence of each carbon source and the maximum absorbance was measured and compared to the chromate-free controls. Estimated IC50s were calculated using a linear regression equation determined by log Cr(VI) concentrations versus percent growth inhibition (as measured by differences in maximum optical densities between the Cr(VI)-containing and Cr(VI)-free treatments).

2.3.5. Lag time calculations

Lag times were estimated using a modified Gompertz equation [51] and optical density data (Eq. (2)) where lag time (γ) in which N is the optical density when growth is first observed at time (t), N_0 is the optical density at time zero, μ_{max} is the maximum growth rate, and A is equal to $\ln(N_{\text{st}}/N_0)$ where N_{st} is the optical density when the culture reaches steady state.

$$\ln \frac{N}{N_0} = A \exp \left\{ -\exp \left[\frac{\mu_{\text{max}} \cdot e}{A} (\gamma - t) + 1 \right] \right\} \quad (2)$$

3. Results and discussion

3.1. Carbon Source Utilization Capabilities

Strain LLW01 utilized a wide variety of carbon sources as an increase in protein was observed for 13 of the 14 potential cellulose degradation products after 72 h (Fig. 1). There was variability in total protein accumulated depending on the carbon source indicating strain LLW01 likely obtained more energy from some carbon sources (such as sucrose and butyrate) compared to others (such as ethanol or xylose) during this time period. No measurable growth was observed after 72 h when formate was the sole carbon source available. These cellulose degradation products are likely present in LLW sites and other soil environments suggesting strain LLW01 may be capable of utilizing any of these carbon sources when available.

3.2. Influence of Carbon Source on Growth in the Presence of Cr(VI)

Glucose, lactate, butyrate, and ethanol were used as representative

carbon sources of various types of potential cellulose degradation products as well as possible energy sources for biostimulation at contaminated sites (i.e., sugars, organic acids, and alcohols). If either glucose or lactate were provided as the sole carbon source, strain LLW01 grew in the presence of 50 μM Cr(VI) (Fig. 2a and b). This is consistent with the conditions under which the organism was isolated. However, if ethanol or butyrate were the sole carbon source, no growth of strain LLW01 was observed in the presence of 50 μM Cr(VI) (Fig. 2c and d), even after 38 days (data not shown). Cr(VI) was more toxic to strain LLW01 in the presence of glucose compared to lactate as evident by reduced extent of growth (maximum OD₆₀₀), slower growth rate, and a lower IC50 for Cr(VI) (Table 1, Fig. S1-S4). In comparison, there was no measurable decrease in the maximum OD₆₀₀ or growth rate of strain LLW01 when lactate and 50 μM Cr(VI) were present. The IC50 for Cr(VI) was also approximately three times higher when lactate was the sole carbon source compared to glucose. Cr(VI) toxicity to strain LLW01 did not correlate with growth rate. In the absence of Cr(VI), the highest growth rate observed was in the presence of glucose, while the lowest was in the presence of lactate (Table 1).

Cr(VI) concentrations below 50 μM Cr(VI) were evaluated to determine at which concentration Cr(VI) becomes toxic to strain LLW01 when either ethanol or butyrate served as the sole carbon source (Fig. S3, S4). Growth was measurable only when Cr(VI) concentrations were below 5 μM Cr(VI) in the presence of ethanol (Fig. S3). The results indicate that the IC50 is less than 5 μM Cr(VI) demonstrating that Cr(VI) is over 30-fold more toxic to strain LLW01 in the presence of ethanol compared to lactate. Butyrate was less toxic than ethanol as only a 28% decrease in max OD was observed in the presence of 5 μM Cr(VI) and no measurable decrease in growth was observed below this concentration. Overall, these results demonstrate that Cr(VI) is significantly more toxic to strain LLW01 when ethanol or butyrate is the sole carbon source compared to glucose or lactate and suggests there is a strong carbon-dependent chromate toxicity mechanism in *Arthrobacter* sp. LLW01.

To our knowledge, this is the first observation of a carbon-dependent chromate toxicity response in an *Arthrobacter* isolate. While the identification of the specific toxicity mechanism or mechanisms is beyond the scope of this study, we can develop hypotheses based on our findings. Evidence of a carbon-dependent toxicity mechanism, rather than general cell damage by Cr(VI), was suggested when a decrease in cell viability was only observed if both ethanol and Cr(VI) were present, indicating their combined presence was necessary for Cr(VI) toxicity. It is possible that the uptake of ethanol by strain LLW01 increased the uptake of Cr(VI) as well leading to this increased toxicity; however, no measurable decrease in Cr(VI) occurred in these treatments. It could be possible that strain LLW01 cannot gain enough energy from carbon sources such as butyrate and ethanol to both grow and mitigate toxicity concurrently, which would support the observation that some of the

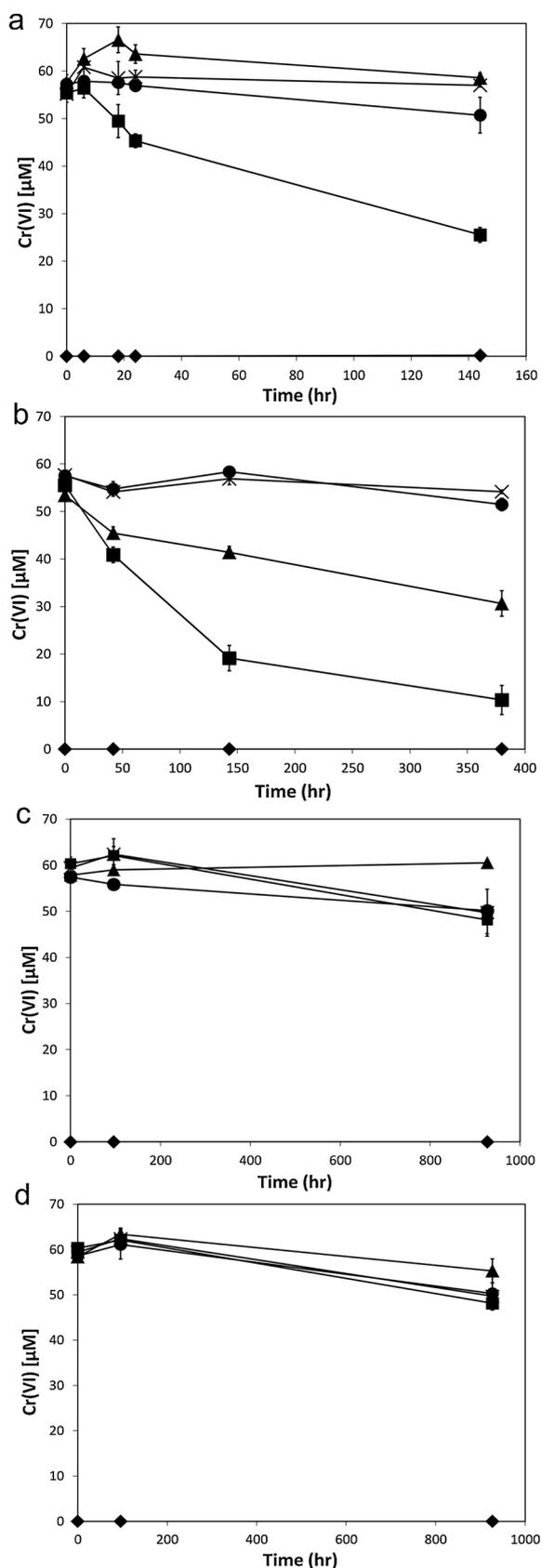


Fig. 3. Cr(VI) concentrations over time when (a) glucose, (b) lactate, (c) ethanol, and (d) butyrate were the sole carbon sources available to *Arthrobacter* sp. LLW01 in the (■) presence of 50 µM Cr(VI). Additional symbols: (◆) CF media only; (●) *Arthrobacter* sp. LLW01 and 50 µM Cr(VI) only; (▲) carbon source and 50 µM Cr(VI) only; (X) 50 µM Cr(VI) only. Error bars represent \pm one standard deviation ($n = 3$) and are smaller than the symbols where not visible.

cells were viable, but did not grow under the conditions presented. This is common in aerobic systems where Cr(VI) reduction is coupled to carbon oxidation of organic compounds [16] and the transfer of electrons to Cr(VI) is inefficient. However, this toxicity can be mitigated if excess carbon is available [16]. It is unlikely cells were energy-limited in this study as excess carbon was added in all experiments at a minimum ratio of 100:1 (carbon to Cr(VI)). When ethanol was present and Cr(VI) concentrations were as low as 5 µM and the carbon to Cr(VI) ratio was more than 1000:1, cell growth still did not occur. Additionally, the highest protein concentrations from the carbon utilization study of these four carbon sources was measured for butyrate (52.18 mg/L) which was significantly higher than both ethanol (t-test; $p = 0.0068$) and glucose (t-test; $p = 0.0266$). Therefore, efficiency in carbon metabolism of butyrate cannot solely explain the higher Cr(VI) toxicity. It is possible that the metabolism of EtOH and butyrate increase the uptake of Cr(VI) by cells which leads to this higher toxicity, but no measurable decrease in Cr(VI) concentration was measured. While we did not conduct any studies to identify the specific toxicity mechanism, toxicity is more likely due to the direct interaction between Cr(VI) and cellular enzymes, such as NADH-dependent enzymes, used in specific carbon metabolism pathways. Similar interactions have been described for other metals and cellular enzymes such as in the case of aerobic PQQ-dependent toxicity of uranium to a *Pseudomonas* sp. [46,52] suggesting that Cr(VI)-enzyme interactions could be potential mechanisms in strain LLW01 as well. Future studies will aim to identify the specific mechanism or mechanisms that lead to this toxicity.

3.3. Influence of Carbon Source on Cr(VI) Reduction

There was an association between Cr(VI) reduction and the capability of strain LLW01 to grow in the presence of 50 µM Cr(VI). When glucose was the sole carbon source, strain LLW01 was capable of growth and Cr(VI) reduction was measured decreasing from 50 µM Cr(VI) to 25 µM Cr(VI) within 144 h (Fig. 3a); the calculated first order reduction rate coefficient was $4.4E-03 \pm 8.8E-04 \text{ h}^{-1}$ ($n = 3$). Most of this reduction occurred after 18 h when the cells were in stationary phase indicating the cells first grew exponentially then Cr(VI) reduction began.

In the presence of lactate and strain LLW01 Cr(VI) concentrations decreased by 80% after 380 h (Fig. 3b). However, there was significant abiotic Cr(VI) reduction in controls containing lactate and Cr(VI) suggesting that half of the reduction in Cr(VI) concentration was due to abiotic reduction. This was likely due to the formation of a lactate-Cr(VI) complex or sodium lactate syrup impurities that were acting as additional reductants as Brodie et al. [36] observed. In controls with lactate, approximately 40% of the Cr(VI) present was no longer measurable using the DPC assay and this decrease was observed consistently over multiple studies. Therefore, the net reduction attributed to the presence of strain LLW01 was about 40% or 20 µM Cr(VI) over the 380 h period, most of which occurred in the first 160 h while the cells were in exponential phase. This contrasts with strain LLW01 grown with glucose when Cr(VI) was reduced during stationary phase. The calculated first order Cr(VI) reduction rate coefficient with lactate was $5.0E-03 \pm 1.2E-03 \text{ h}^{-1}$. Therefore, Cr(VI) reduction rates were not significantly different (t-test, $p = 0.7037$) regardless of whether glucose or lactate were present but there was a difference in the time that had passed before Cr(VI) reduction began. When either butyrate or ethanol were the sole carbon sources, no significant reduction of Cr(VI) was measured over a period of 38 days (Fig. 3c and d). ICP-MS analyses suggest that the measured decrease in Cr(VI) concentration was due to the reduction of Cr(VI), rather than biosorption or general cellular uptake (Fig. S5) indicating strain LLW01 is capable of contributing to Cr(VI) reduction, likely forming Cr(III) [53] under the provided conditions. It is possible Cr(VI) was reduced to soluble organo-Cr(III) products as there was no measurable difference in the filtered total Cr over the course of the experiments [54,55].

Reduction of Cr(VI) occurred only when *Arthrobacter* sp. LLW01 grew in this study suggesting Cr(VI) reduction and cell growth was associated. The use of a variety of carbon sources allowed us to observe a host of complex responses that would not be possible by using glucose alone. While Cr(VI) reduction occurred under many conditions, to observe any measurable growth when ethanol was present, the Cr(VI) concentration had to be below 5 μM Cr(VI). This concentration is close to the U.S. EPA Drinking Water Standard of 0.1 mg total Cr L⁻¹ (100 ppb or approximately 2 μM Cr) (Environmental Protection Agency, 2016), and above the newly adopted maximum contaminant level for chromium (10 ppb) in the State of California (State Water Resources Control Board, California Environmental Protection Agency, 2016). Therefore, bioremediation efforts with strain LLW01 would be ineffective if ethanol or butyrate were supplied to enhance Cr(VI) reduction. However, if glucose or lactate were supplied then strain LLW01 may be able to contribute to the *in situ* remediation of Cr(VI) more effectively.

3.4. Cr(VI) Toxicity Mitigation by Sulfate

Chromate can be taken up by the cell via the same mechanism as sulfate [48] and therefore, it may be possible to mitigate toxicity of chromate via competitive exclusion by sulfate. In this study, the addition of excess sulfate in the presence of Cr(VI) led to an increase in cell densities, but only mitigated toxicity to a small extent (Fig. 4). The maximum OD₆₀₀ in the presence of 5 mM SO₄²⁻ increased 18% compared to when SO₄²⁻ was absent. This increase in growth was still only half the maximum growth measured in the absence of Cr(VI). Cr(VI) reduction by strain LLW01 was not significantly different with the addition of 5 mM SO₄²⁻ (Fig. S6). Overall, these results indicate that the addition of excess SO₄²⁻ mitigated Cr(VI) toxicity to a small extent, but no difference in Cr(VI) reduction was observed (Fig. S4). It also suggests that potential machinery in sulfate transport is not likely playing a role in Cr(VI) toxicity to strain LLW01 observed here and introduces questions about whether competitive exclusion, by sulfate or other soil constituents, will aid in bioremediation efforts by strain LLW01.

3.5. Cell Viability in the Presence of Cr(VI)

A cell viability study with ethanol as the sole carbon source was conducted to determine whether the lack of growth by strain LLW01 in the presence of Cr(VI) was due to a bactericidal (causing cell death) or

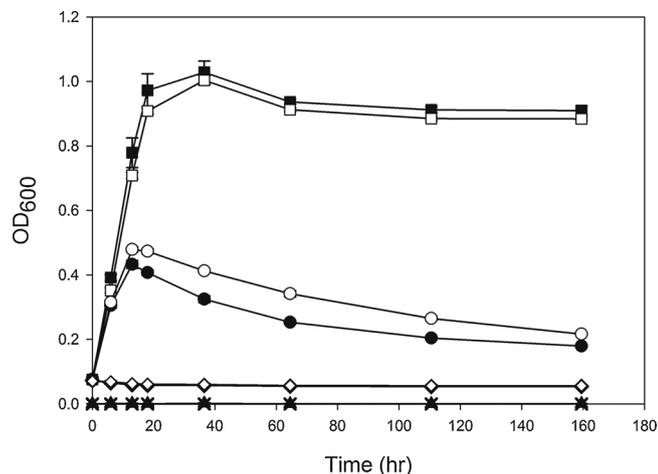


Fig. 4. Chromate toxicity mitigation by the addition of excess sulfate. Growth of *Arthrobacter* sp. LLW01 with glucose in the (●,○) presence and (■,□) absence of 50 μM Cr(VI). Open symbols indicate the additional presence of 5 mM sulfate (SO₄²⁻). Additional symbols: (▲) CF medium only; (◆) *Arthrobacter* sp. LLW01 only; (X) Glucose only. Error bars represent \pm one standard deviation (n = 3) and are smaller than the symbols where not visible.

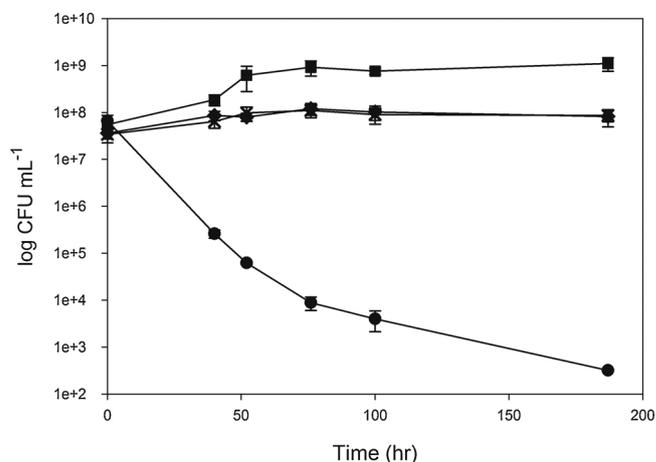


Fig. 5. Cell viability of *Arthrobacter* sp. LLW01 when ethanol (EtOH) was the sole carbon source in the (●) presence and (■) absence of 50 μM Cr(VI). Additional symbols: (◆) *Arthrobacter* sp. LLW01; (X) *Arthrobacter* sp. LLW01 and 50 μM Cr(VI). Plate count data representing cell viability are reported as log CFU mL⁻¹. Error bars represent \pm one standard deviation (n = 5) and are smaller than the symbols where not visible.

bacteriostatic (causing growth inhibition) response to Cr(VI). As ethanol had the greatest effect on chromate toxicity, it was used in this cell viability study. Results indicate that while the majority of cells were no longer viable, some cells from the treatment containing ethanol, strain LLW01, and 50 μM Cr(VI) remained viable after 187 h (3.2 \times 10² CFU mL⁻¹, Fig. 5). These results indicate the lack of cell growth and Cr(VI) reduction may be reversible even though an approximate five-order magnitude loss of cell viability was observed after 8 days. This suggests that a switch to a more favorable carbon source either intentionally through biostimulation efforts or due to the production of additional cellulose degradation products *in situ* could stimulate the growth of strain LLW01 and subsequent reduction of Cr(VI). Notably, this decrease in cell viability was only observed when both ethanol and 50 μM Cr(VI) were present as there was no significant change in cell viability in the carbon-free control containing strain LLW01 and 50 μM Cr(VI). This suggests the combination of carbon source and chromate was necessary for cell death to occur which indicates cells were more susceptible when metabolically active. As previously mentioned, the uptake of ethanol may increase the uptake of Cr(VI) and contribute to the toxicity observed.

4. CONCLUSIONS

Results of this study demonstrate that carbon metabolism affects *Arthrobacter* sp. LLW01 growth in the presence of Cr(VI) and the subsequent reduction of Cr(VI) which would have detrimental effects on bioremediation efforts involving this organism. While it is possible to limit the intentional addition of ethanol and butyrate into the environment to stimulate Cr(VI) remediation, natural attenuation methods will likely fail if these carbon sources are the primary sources available. Therefore, successful Cr(VI) remediation by *Arthrobacter* sp. LLW01 would require the addition of sugars such as glucose and lactate while limiting the availability of alcohols and some organic acids such as ethanol and butyrate. Soil systems are complex and the role of other soil constituents cannot be overlooked. Additional studies are needed to incorporate other constituents that could affect Cr(VI) reduction to integrate these results into large-scale bioremediation strategies. The results presented here should be useful in developing *in situ* bioremediation strategies and in understanding how *Arthrobacter* spp. may affect these strategies.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jhazmat.2018.05.020>.

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