Appl Microbiol Biotechnol (2008) 78:1007–1016
DOI 10.1007/s00253-008-1381-x

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Hexavalent chromium reduction in *Desulfovibrio vulgaris* Hildenborough causes transitory inhibition of sulfate reduction and cell growth

A. Klonowska · M. E. Clark · S. B. Thieman · B. J. Giles · J. D. Wall · M. W. Fields

Received: 14 November 2007 / Revised: 7 January 2008 / Accepted: 18 January 2008 / Published online: 12 February 2008
© Springer-Verlag 2008

Abstract *Desulfovibrio vulgaris* Hildenborough is a well-studied sulfate reducer that can reduce heavy metals and radionuclides [e.g., Cr(VI) and U(VI)]. Cultures grown in a defined medium had a lag period of approximately 30 h when exposed to 0.05 mM Cr(VI). Substrate analyses revealed that although Cr(VI) was reduced within the first 5 h, growth was not observed for an additional 20 h. The growth lag could be explained by a decline in cell viability; however, during this time small amounts of lactate were still utilized without sulfate reduction or acetate formation. Approximately 40 h after Cr exposure (0.05 mM), sulfate reduction occurred concurrently with the accumulation of acetate. Similar amounts of hydrogen were produced by Cr-exposed cells compared to control cells, and lactate was not converted to glycogen during non-growth conditions. *D. vulgaris* cells treated with a reducing agent and then exposed to Cr(VI) still experienced a growth lag, but the addition of ascorbate at the time of Cr(VI) addition prevented the lag period. In addition, cells grown on pyruvate displayed more tolerance to Cr(VI) compared to lactate-grown cells. These results indicated that *D. vulgaris* utilized lactate during Cr(VI) exposure without the reduction of sulfate or production of acetate, and that ascorbate and pyruvate could protect *D. vulgaris* cells from Cr(VI)/Cr (III) toxicity.

Keywords *Desulfovibrio* · Chromium · Cell toxicity

J.D. Wall and M.W. Fields are both affiliated to the Virtual Institute of Microbial Stress and Survival (http://vimss.lbl.gov/).

M.E. Clark and S.B. Thieman contributed equally to this work.

A. Klonowska · M. E. Clark · S. B. Thieman
Department of Microbiology, Miami University,
Oxford, OH, USA

M. E. Clark · M. W. Fields (✉)
Center for Biofilm Engineering, Montana State University,
366 EPS Bldg,
Bozeman, MT 59717, USA
e-mail: matthew.fields@erc.montana.edu

B. J. Giles · J. D. Wall
Department of Biochemistry, University of Missouri,
Columbia, MO, USA

J. D. Wall
Department of Molecular Microbiology & Immunology,
University of Missouri,
Columbia, MO, USA

M. W. Fields
Department of Microbiology, Montana State University,
Bozeman, MT, USA

Present address:
A. Klonowska
Laboratoire des Symbioses Tropicales et Méditerranéennes,
Institut de Recherche pour le Développement,
UMR 113,
Montpellier, France

Introduction

Chromium is a common contaminant of both soil and water and is considered both a carcinogen and a mutagen. Chromium is the third most common pollutant at hazardous waste sites and the second most common inorganic contaminant after Pb. Cr(VI) is more soluble than Cr(III) and is considered to be 1,000-fold more toxic than Cr(III) in humans (ATSDR 2004). The U.S. Environmental Protection Agency estimates that approximately 2,840 metric tons of total chromium is emitted annually into the atmosphere in the United States. Cr(VI) can easily pass through cell membranes where it can be reduced to reactive species

[Cr(III) and Cr(V)] and can generate free radicals that can damage nucleic acids and proteins. It is typically assumed that the reduction to Cr(III) renders Cr less soluble and less toxic and is an advantageous speciation to maintain within contaminated soil and water (Cheung and Gu 2007).

Many U.S. Department of Energy field sites are contaminated with mixtures of metals and radionuclides as well as nitrate and hydrocarbons (Riley and Zachara 1992). Mixed wastes are difficult and expensive to remediate effectively with current physical and chemical technologies, but bioremediation with indigenous microorganisms holds promise as a cost-effective and comparatively unobtrusive technology for in situ remediation (Iwamoto and Nasu 2001). Although many microorganisms have demonstrated an ability to reduce Cr(VI) (Cheung and Gu 2007), of particular interest are the metal reduction capabilities of sulfate-reducing bacteria (SRB) that result from both direct (cell-mediated) and indirect (sulfides) reactions. *Desulfovibrio vulgaris* Hildenborough (ATCC 2597) is a model SRB and has been shown to reduce metals, metalloids, and radionuclides (Heidelberg et al. 2004). The cell-mediated reduction involves hydrogenases and cytochrome c_3 as well as reduction by hydrogen sulfide (Lovley and Phillips 1994; Chardin et al. 2002).

Previous work with purified enzymes and whole cells has shown that *D. vulgaris* Hildenborough is capable of reducing Cr(VI), but cells are unable to use Cr(VI) as a terminal electron acceptor linked to growth (Chardin et al. 2002). Because the end product of sulfate reduction, sulfide, can directly reduce many heavy metals [e.g., Cr (VI)], it is difficult to discriminate the biological effects of heavy metal exposure on sulfate-reducing bacteria. In the described experiments, cells were washed anoxically to reduce the carry-over sulfide so that cellular responses could be observed. Other investigators have used similar methodology to elucidate the biological effects of heavy metals (Chang et al. 2004). We report that Cr(VI) exposure decouples lactate consumption from sulfate reduction and acetate production. In addition, we show that the loss of viability is not a consequence of the loss of reducing conditions, but appears to be due to Cr(III) toxicity.

Materials and methods

Bacterial strains and growth conditions

Cultures of *D. vulgaris* Hildenborough (ATCC 29579) were obtained from Dr. T.C. Hazen (Lawrence Berkeley National Laboratory). Media were prepared anoxically by boiling under oxygen-free N_2 gas and dispensed into gassed tubes that were sealed with butyl rubber stoppers. Modified LS4D is LS4D medium used as previously described (Clark et al. 2006) except the $NaC_3H_6O_3$ and Na_2SO_4 concentrations which were 45 and 30 mM, respectively. Lactate serves as the carbon and energy source. As previously described, the medium was not prepared with a reducing agent (Clark et al. 2006). The use of resazurin in the culture medium served as a general indicator of oxidative–reductive potential (ORP), and all cultures were incubated at 30°C.

Cells that served as inocula were grown in 7 ml of LS4D medium under an oxygen-free N_2 atmosphere until an optical density (600 nm) between 0.5 and 0.7 was reached. Cells were harvested by centrifugation at 2,000×*g* at room temperature for 10 min under anoxic conditions. The supernatant was aspirated under a N_2 atmosphere, the cell pellets washed once in fresh LS4D medium (equal volume), and the cell pellet was then re-suspended in fresh LS4D medium (1 ml). The cell suspension was inoculated immediately into fresh LS4D medium to an initial OD_{600} of approximately 0.08. Exposure to oxygen during transfer to new tubes was avoided by gassing sterile syringes with sterile N_2. Cr(VI) (potassium chromate) was added to cultures to obtain final concentrations of approximately 0, 0.02, 0.05, and 0.1 mM, and zero-time samples were immediately withdrawn and analyzed. Samples of 150 to 300 µl were recovered with a N_2-flushed syringe every hour for the first 3 h, and then approximately every 5 h. Samples were analyzed for lactate, acetate, ethanol, sulfate, pyruvate, Cr(VI), and optical density. Growth experiments and Cr(VI) exposure were done in triplicate.

Analytical techniques

Lactate, acetate, and sulfate concentrations were measured by ion chromatography (Metrohm-Peak) with a Metrosep organic acid column and a Metrosep Anion Supp 5 column, respectively. Pyruvate concentrations were measured via HPLC (Shimadzu LC10Ai) connected to a Waters 484 detector with a BioRad HPLC Organic Acid Analysis Column. Intracellular pyruvate levels of concentrated cell lysates were determined with an enzyme-based pyruvate assay kit (BioVision, Inc.) following the manufacturer's instructions. H_2 gas production was assessed with a Varian CP3380 GC and a Varian packed molecular sieve 5a column under N_2 gas. Protein levels were determined by the Lowry method and bovine serum albumin (Pierce Biochemicals) as a standard. Carbohydrate was determined with a cysteine–sulfuric acid colorimetric assay as previously described (Chaplin 1986). All assays were done in duplicate. Ethanol was measured via an EnzyChromTM Ethanol Assay Kit (BioAssay Systems) following the manufacturer's instructions. Oxidation–reduction potential was measured via a HQ20 probe (Hach). A colorimetric assay was used to measure sulfide levels. Samples were taken anoxically via syringe and filtered (0.22-µm PVDF filter). A

50-μl aliquot of the filtered sample was immediately mixed with 4 ml of copper reagent (5 ml 5 M HCl, 0.62 g $CuSO_4$ in 500 ml dH_2O) and absorbance measured (480 nm). Sodium sulfide prepared under anoxic conditions was used as the standard.

Cr(VI) reduction analysis

The Cr(VI) levels were determined by adding 0.1 ml of filtered sample (0.22 μm pore-sized filters) to 0.9 ml of Chroma Ver3 (Hach) solution as described by Viamajala et al. (2002). The mix was then incubated for 15 min at room temperature, and absorbance was measured at 540 nm. A Cr(VI) calibration curve was obtained in the range 0 to 0.12 mM Cr(VI) with potassium chromate as a standard. The Cr(VI) levels measured in the bacterial cultures were compared to those in un-inoculated medium and in cultures that contained heat-killed cells. Cr(VI) concentrations did not change significantly during the experimental period in un-inoculated medium or in cultures of heat-killed cells.

Reducing agents

The effect of reducing agents on Cr(VI) exposure was determined by the addition of titanium citrate, cysteine, or sodium sulfide. Cells were cultivated and washed as described above and used to inoculate fresh LS4D to approximately 0.07 OD_{600}. Each tube (10 ml LS4D medium) had a final concentration of either 1.6 mM sodium sulfide, 0.5 mM cysteine, or 0.002% (wt/vol) Ti-citrate. No addition of reducing agent served as a control. Immediately after the addition of the reducing agent, tubes received a final concentration of 0.05 mM Cr(VI). Samples were withdrawn immediately and at appropriate intervals to measure Cr(VI) and growth. Samples were done in duplicate and the entire experiment was repeated several times.

The effect of ligand–Cr(III) complexes on washed *D. vulgaris* cells was also investigated. As described above, washed cells were used to inoculate fresh LS4D to an OD_{600} of approximately 0.07. Separately, the cultures received final concentrations of either 0.05 mM Cr(VI), 0.05 mM Cr(VI) and 0.05 mM sodium ascorbate (with ascorbate added first), 0.05 mM Cr(VI) and 0.05 mM sodium ascorbate 3 h after Cr (VI) exposure, 0.05 mM sodium ascorbate, a 48-h ascorbate–Cr(III) complex, or no addition as a control. Cultures were monitored for growth over time and each condition was tested in duplicate and repeated several times.

The effects of various concentrations of sodium ascorbate on the growth effects of Cr(VI) were also tested. Cultures were started at an OD_{600} of approximately 0.07 with washed cells. Sodium ascorbate was added to cultures at the following final concentrations: 0, 0.05, 2, 5, 10, 25, and 50 mM. Immediately after the addition of sodium ascorbate, 0.05 mM Cr(VI) was added to each tube (final concentration) and growth was monitored. The experiment was carried out in duplicate.

The temporal sensitivity of organic complexation of the chromium species was tested by the addition of 0.05 mM sodium ascorbate at various times post-Cr(VI) exposure. Cultures were started with washed cells to an OD_{600} of 0.07. Cr(VI) was added to a concentration of 0.05 mM, and 0.05 mM sodium ascorbate was added in 15-min intervals starting at time 0 up to 3 h. Growth was monitored over time and conditions were tested in duplicate.

Viability

Cell viability was analyzed by MPN (most probable number) analysis at 0, 10, and 20 h post-Cr(VI) addition. Briefly, washed cells were inoculated into fresh medium to an OD_{600} of 0.05. The Cr(VI) was added to a final concentration of 0.05 mM, and 1 ml was immediately withdrawn for serial dilutions into pre-reduced medium. Dilution series were also conducted for the 10- and 20-h time points, and all dilutions were done in triplicate.

To determine the capacity of medium from Cr(VI)-exposed cultures to support growth, washed cells were inoculated into fresh LS4D medium to an OD_{600} approximately 0.07, and 0.05 mM Cr(VI) was added. At 10 h post-exposure, a 10-ml sample of the culture was taken with a syringe and cells removed by filtration. The filtrate was collected directly into a sterile, N_2 gassed tube and inoculated with freshly washed cells to an OD_{600} of 0.05 and growth was monitored. Concurrently, cells that had been exposed to 0.05 mM Cr(VI) for 10 h were pelleted via centrifugation at 2,000×*g* for 10 min at room temperature. The supernatant from these cultures was removed, and the cell pellets were re-suspended in fresh LS4D medium.

Results

ORP in the absence and presence of Cr(VI)

Four replicate subcultures of *D. vulgaris* Hildenborough were made from exponential-phase cultures. The inocula were washed once anoxically in sterile medium to remove carry-over sulfides that would interfere with cell responses to Cr (VI). The ORP was determined for un-inoculated medium (without reducing agent) and for medium that contained washed cells. The ORP for medium alone was −60±18 mV. The addition of 0.02 mM Cr(VI) to medium (without cells) increased the ORP from −60±18 to −10±15 mV, and higher Cr(VI) levels (i.e., 0.05 and 0.1 mM) altered the ORP to −9.0±11 and +6.3±10 mV, respectively. These results

indicated that doubling the Cr(VI) concentrations caused relatively small changes in the ORP.

The ORP for medium inoculated with washed cells was approximately −200 mV. The drop in ORP was most likely due to residual hydrogen sulfide associated with the cells (approximately 1.5 mM). It is important to note that cells without Cr(VI) or with 0.02 mM Cr(VI) both lagged approximately 1 h prior to growth initiation even though ORP values were different. In addition, the initial ORP for medium inoculated with 0.05 mM Cr(VI) and washed cells was −121±9 mV. The data indicated that the cells could quickly recover from the cell washing preparation and that the ORP after the addition of washed cells and Cr(VI) only varied from approximately −120 to −105 mV. Typically, it is assumed that an ORP value of −100 mV is needed for SRBs to initiate growth (Postgate 1984), and these results indicate that the observed growth lags were Cr(VI) dependent and not a direct result of higher ORP levels.

D. vulgaris ATCC 29579 growth in the absence and presence of Cr(VI)

In LS4D medium without Cr(VI), *D. vulgaris* cells had a growth rate of 0.11±0.01 h^{-1}. When exposed to 0.02 mM Cr(VI), cultures displayed little effect on growth rate, but 0.05 mM Cr(VI) caused an approximate 30-h lag without a significant change in growth rate once growth was initiated (Fig. 1). When cells were exposed to an initial Cr(VI) concentration of 0.1 mM, the *D. vulgaris* cells lagged for approximately 60 h before growth was resumed at a slower rate (μ=0.08±0.01 h^{-1}). It should be noted that the ORP indicator, resazurin, was colorless within 4 and 30 h of the

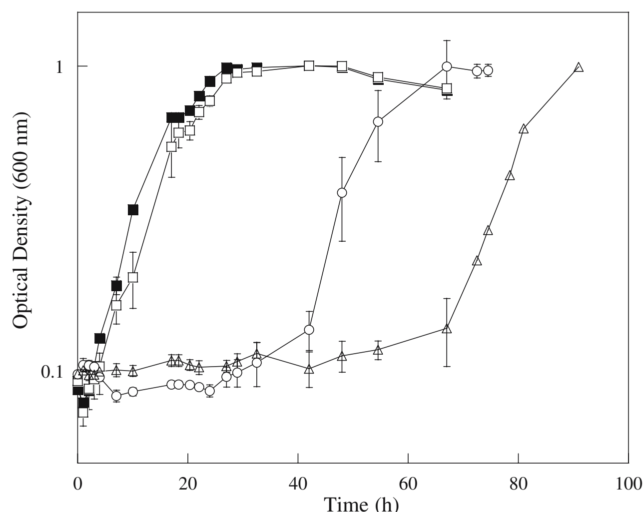

Fig. 1 Growth of *D. vulgaris* cells grown in the absence (*filled square*) or presence of Cr(VI). Washed cells were inoculated to a starting OD_{600} between 0.08 and 0.1 and exposed to 0.02 mM (*empty square*), 0.05 mM (*empty circle*), and 0.1 mM (*empty triangle*) Cr(VI). Cell growth was monitored over time via the OD_{600}

0.05 and 0.1 mM Cr(VI) treatments, respectively, yet the cells continued to lag. These results also suggested that factors other than ORP contributed directly to the growth lag. The final biomass level was not affected at any of the Cr(VI) levels.

Viability

During the approximately 24-h lag period after 0.05 mM Cr (VI) exposure, the cell viability steadily decreased. The viable cell number declined approximately fourfold after 10 h post-exposure (2.4×10^7 to 6.2×10^6 cells/ml) and declined approximately 40-fold after 20 h post-exposure (2.4×10^7 to 6.1×10^5 cells/ml). The decline in viability corresponded to the lag period observed for the 0.05 and 0.10 mM Cr(VI) treatments. These results indicated that cell death and not metabolic stasis could explain the majority of the growth lag.

Lactate, acetate, and sulfate levels

To assess metabolic responses during Cr(VI) exposure, lactate, acetate, and sulfate concentrations were determined during approximately 100 h post-exposure to Cr(VI) (Fig. 2). When exponentially growing *D. vulgaris* cells were washed and inoculated into LS4D medium, the cells initiated growth within 1 h, and lactate and sulfate simultaneously declined (Fig. 2a). When washed *D. vulgaris* cells were exposed to 0.02 mM Cr(VI), the culture still initiated growth within 1 h of exposure, and lactate and sulfate were consumed concomitantly (Fig. 2b). However, the appearance of acetate was not detected for several hours (ca. 5 to 7 h) as opposed to 2 h in the culture without Cr (VI) addition (Fig. 2a and b). When the *D. vulgaris*-washed cells were exposed to 0.05 mM Cr(VI), cell growth lagged for approximately 30 h (Fig. 2c). During the lag, sulfate levels did not decline, but lactate was depleted at a slow rate (0.3 mM/h). When growth resumed, lactate was depleted at a faster rate (1.0 mM/h), sulfate was consumed, and acetate accumulated. However, acetate was not detected until 5 h after growth had been initiated. A 0.1-mM Cr(VI) addition to washed cells caused a growth lag for approximately 60 h, during which sulfate was not consumed but approximately 10 mM lactate was utilized (Fig. 2d). The results indicated that sulfate reduction was transiently decoupled from lactate utilization upon Cr(VI) exposure for *D. vulgaris* cells.

Cr(VI) levels during growth

Depending on the ability of cells to reduce Cr(VI), the growth lag could be a consequence of residual Cr(VI). Cr (VI) concentrations declined to non-detectable levels within

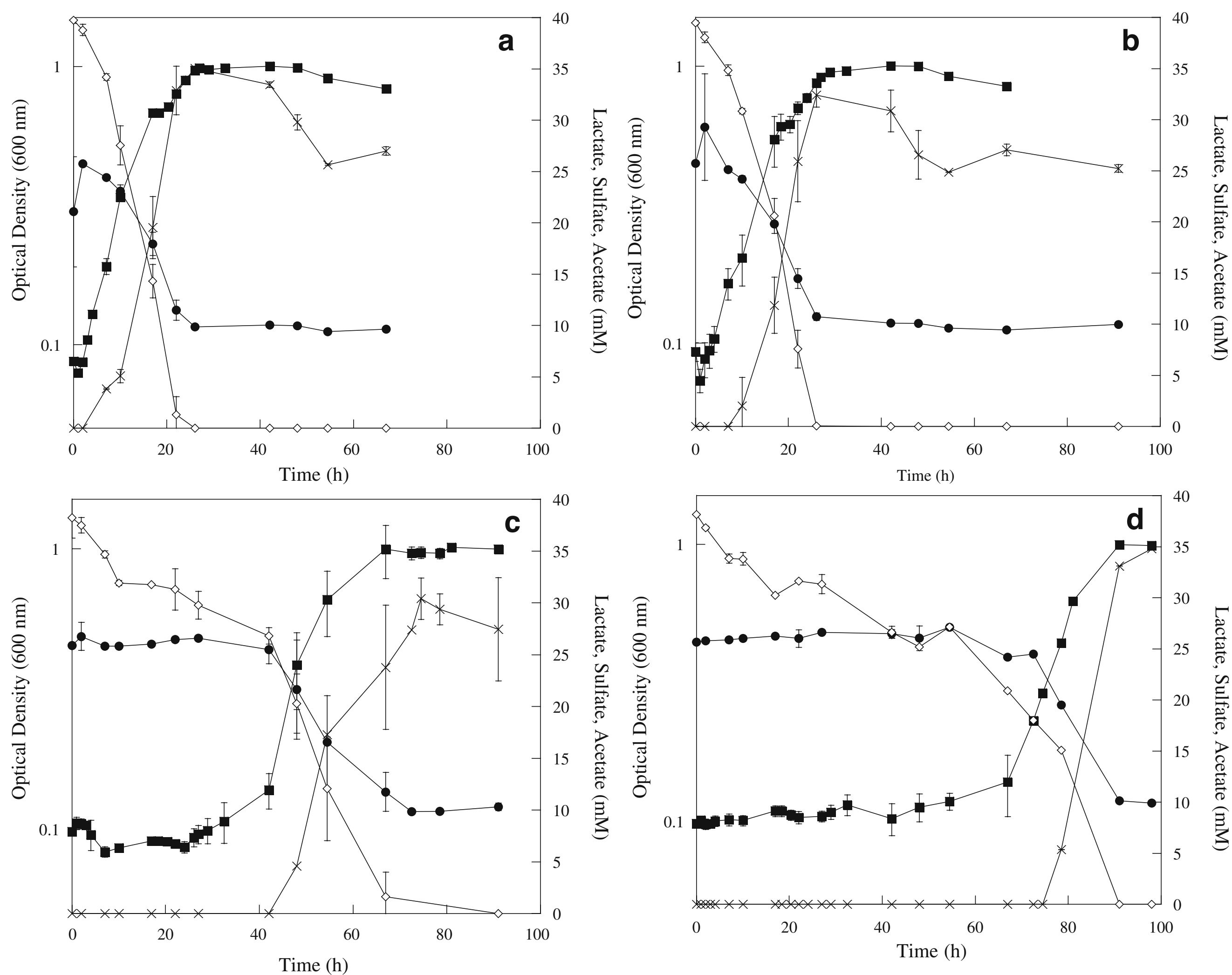

Fig. 2 Substrate analysis of washed *D. vulgaris* cells exposed to Cr (VI). Washed cells were inoculated to an OD_{600} (*filled square*) of approximately 0.08 and lactate (*empty diamond*), sulfate (*filled circle*), and acetate (*ex mark*) levels were monitored over time for control (**a**), 0.02 mM Cr(VI) (**b**), 0.05 mM Cr(VI) (**c**), and 0.1 mM Cr(VI) (**d**)

1 h when cells were exposed to 0.02 mM (data not shown). When *D. vulgaris* cells were exposed to 0.05 mM Cr(VI), the Cr(VI) levels declined almost tenfold within the first 2 h and were not detectable after 5 h (Fig. 3). With 0.1 mM Cr (VI), washed *D. vulgaris* cells decreased the Cr(VI) concentration threefold within 5 h. The Cr(VI) was not detectable after 15 h (Fig. 3). Heat-killed cells adsorbed some Cr(VI), but without biological activity over 90% of the Cr(VI) remained during the same time periods (data not shown). The data indicated that the majority of Cr(VI) was quickly reduced when exponentially growing cells were used, that the process was biological, that growth was not needed for Cr(VI) reduction, and that the cell growth still lagged for considerable time periods even after the Cr(VI) was reduced. These results suggested that the cells were responding to direct and/or indirect consequences of Cr(VI) reduction and not necessarily Cr(VI) per se.

Carbohydrate levels

To identify the product(s) generated from the lactate that was consumed in the absence of sulfate reduction or apparent cell growth, carbohydrate levels were compared in Cr(VI)-treated versus non-treated *D. vulgaris* cells. The partially oxidized carbon could be used to form glycogen, and previous work has shown glycogen formation in *Desulfovibrio* spp. (Santos et al. 1993; Clark et al. 2006). Washed *D. vulgaris* cells were exposed to 0.05 mM Cr(VI) as described above, and both total protein and carbohydrate were determined over a 24-h time period. Cells with or without Cr(VI) had similar carbohydrate to protein ratios initially, and the ratio increased over time in cultures without Cr(VI) whereas the Cr(VI)-treated cells maintained a constant carbohydrate to protein ratio that did not change significantly over the tested time period (Fig. 4a). These

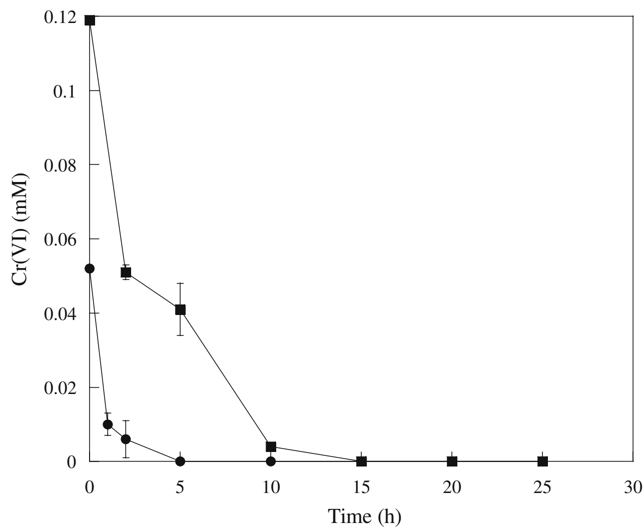

Fig. 3 Reduction of Cr(VI) concentrations over time by washed *D. vulgaris* cells under growth conditions. Cells were exposed to 0.05 mM (*filled circle*) and 0.10 mM (*filled square*) Cr(VI). The presence of Cr(VI) within the supernatant was monitored colorimetrically for 25 h post-exposure

results indicated that during the corresponding lag for the 0.05 mM Cr(VI) treatment cellular carbohydrate levels did not increase.

Hydrogen and pyruvate levels

Incomplete oxidation of lactate could result in an accumulation of acetate, pyruvate, ethanol, and/or hydrogen. When hydrogen levels were measured for *D. vulgaris* cells exposed to 0.05 mM Cr(VI) and compared to non-treated cells, the total hydrogen levels were similar (Fig. 4b). The non-treated cells displayed an accumulation of hydrogen gas that peaked in mid-exponential-phase growth. The Cr (VI)-treated cells accumulated a similar level of hydrogen, and detectable hydrogen levels were measured shortly before growth ensued. In addition, acetate levels did not accumulate until after growth was initiated (Fig. 2c) and neither pyruvate nor ethanol was detected in the culture supernatant (data not shown). Cells were concentrated during the Cr-induced lag phase and cell lysates were obtained. The lysates of the Cr-exposed cells did not have significantly elevated levels of pyruvate compared to non-exposed cells.

Reduced versus complexed Cr

To delineate between the effects of direct Cr exposure and the process of Cr(VI) reduction, Cr(VI) was added to pre-reduced medium using different reducing agents (i.e., sulfide, cysteine, titanium, ascorbate) with and without cells. The addition of the reducing agents significantly lowered the ORP for medium alone (between −300 and −400 mV), and the addition of the reducing agents to a 0.05- or 0.1-mM Cr(VI) solution immediately reduced more than 90% of the Cr(VI) (data not shown). When sulfide, cysteine, or titanium (Ti-citrate) was added alone to washed cells, growth was similar to the control (e.g., no reducing agent), and a lag was not observed (Fig. 5). However, when 0.05 mM Cr(VI) was added to the medium that contained one of the reducing agents, the cells still lagged for approximately 30 h (Fig. 5). These results indicated that the cells were susceptible to Cr(VI) exposure even when the Cr(VI) was added to pre-reduced medium and that by-products or intermediates (i.e., cell component complexes or reduced intermediates, respectively) from Cr(VI) reduction were likely toxic to the cells.

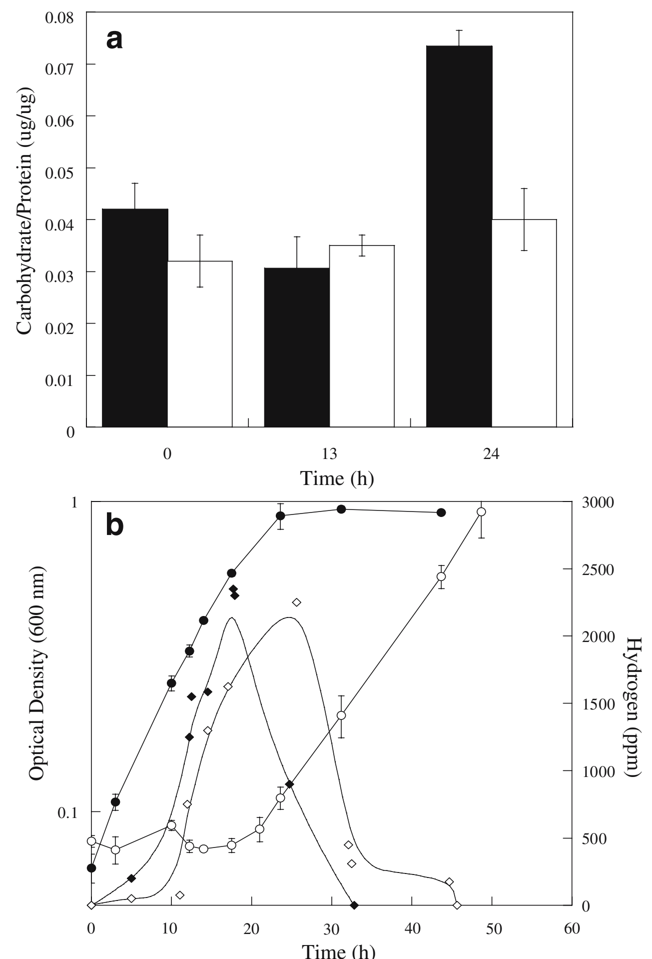

Fig. 4 Determination of cellular carbohydrate to protein ratio in *D. vulgaris* cells during the Cr-induced lag period (**a**). Carbohydrate to protein ratios (microgram per microgram) for controls cells not exposed to Cr(VI) (*black bars*) and cells exposed to 0.05 mM Cr (VI) (*white bars*). Hydrogen production during the 30-h lag period measured via gas chromatography (**b**). Washed cells in the absence of Cr(VI) were monitored for growth (*filled circle*) and hydrogen production (*filled diamond*) as a control. Washed cells were also exposed to 0.05 mM Cr(VI) and monitored for growth (*empty circle*) and hydrogen production (*empty diamond*)

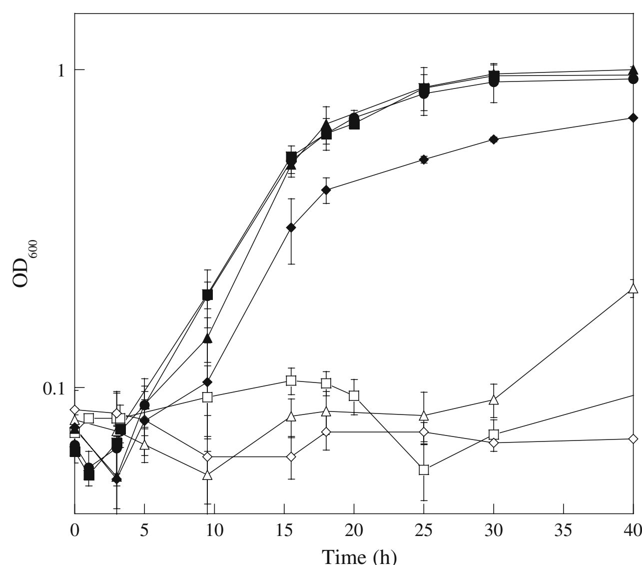

Fig. 5 Regeneration of reducing conditions during Cr(VI) exposure. Washed cells were inoculated into LS4D that contained 0 mM Cr(VI) (*filled circle*), 1.5 mM sodium sulfide (*filled square*), 0.05 mM Cr(VI) + sodium sulfide (*empty square*), 0.002% Ti-citrate (*filled triangle*), 0.05 mM Cr(VI) + Ti-citrate (*empty triangle*), 0.05 mM cysteine (*filled diamond*), and 0.05 mM Cr(VI) + cysteine (*empty diamond*). Cultures were monitored over time for growth via OD_{600}

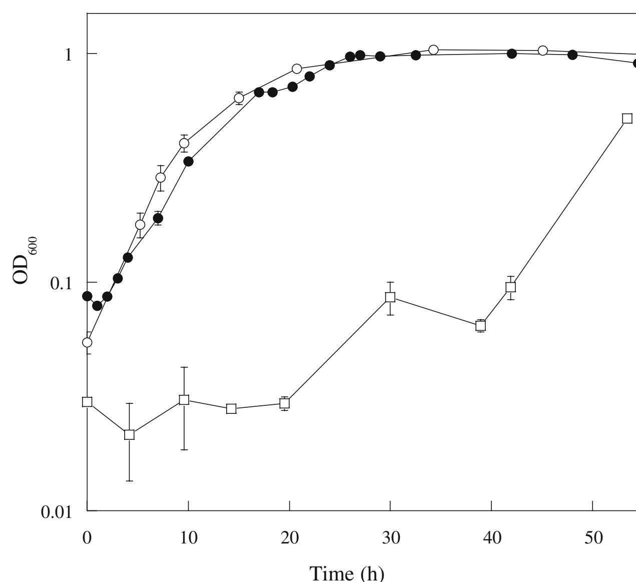

Fig. 6 Analysis of supernatant and cell pellets after 10 h Cr(VI) exposure. Filtered supernatants were inoculated with washed *D. vulgaris* cells, and cell pellets (Cr exposed) were re-suspended into fresh LS4D medium. Growth was monitored over time via OD_{600}. Control cells (*filled circle*) were washed and inoculated into LS4D without Cr(VI), and *empty circle* represents the growth of freshly washed cells (no previous Cr exposure) in the spent supernatant collected 10 h post-Cr exposure (0.05 mM) and *empty square* represents the cell pellet of Cr-exposed cells (10 h) that was re-suspended into fresh LS4D

To determine if the culture supernatant contained soluble Cr intermediates that inhibited growth, cells that were 10 h post-Cr exposure were centrifuged and re-suspended into fresh medium (no Cr). The culture supernatant was also collected anoxically, filtered, and re-inoculated with cells that had not been exposed to Cr(VI). When fresh cells were inoculated into the spent Cr-culture supernatant, the cells were not affected in growth and did not display a growth lag (Fig. 6). When the Cr-exposed cell pellet was inoculated into fresh medium, the cells lagged and did not grow for 20 h. This, with the additional 10 h post-Cr exposure, coincided with the 30-h lag period initially observed (Fig. 6). These results indicated that the culture supernatant did not contain significant levels of Cr reduction intermediates that were inhibitory to growth and that any inhibitory product was most likely cell associated. It is also possible that the cells were in a physiological state caused by the Cr(VI) exposure that changed over time.

When the medium was pre-reduced with 0.05 mM ascorbate, the ORP declined to −350 mV, the addition of 0.05 mM Cr(VI) did not cause a lag, and cell growth was similar to the control without the Cr(VI) addition (Fig. 7). In addition, when the Cr(VI) was combined with the ascorbate prior to cell exposure, a growth lag was not observed and cell growth was similar to the control. However, if cells were first exposed to the Cr(VI) and then the ascorbate was added later (after 1 h), the cells lagged and grew as if exposed to Cr(VI) only (Fig. 7). The lag could be avoided if the ascorbate was added within 0.5 h of

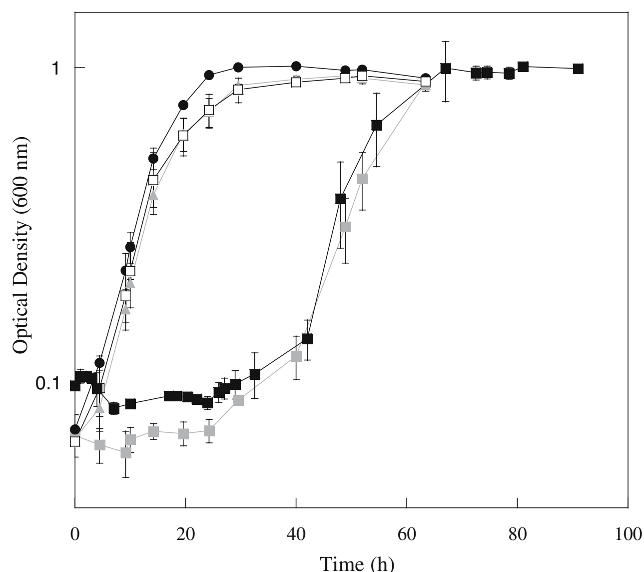

Fig. 7 Growth of Cr(VI) exposed *D. vulgaris* cells in the presence of ascorbate. Washed cells were inoculated into LS4D alone (*filled circle*), with 0.05 mM Cr(VI) (*filled square*), 0.05 mM ascorbate (*empty square*), 0.05 mM Cr(VI) plus 0.05 mM ascorbate 3 h post-Cr (VI) exposure (*gray square*), and 0.05 mM Cr(VI) plus 0.05 mM ascorbate added concurrently (*gray triangle*). Growth was monitored via OD_{600}

the initial Cr(VI) exposure (Fig. 6). In comparison, the addition of reducing agents (i.e., sulfide or Ti-citrate) did not rescue cell growth, and these results suggested that the protection afforded by ascorbate was not merely due to the ability to reduce but complex the Cr.

When cells were grown with pyruvate as the carbon and electron source, the growth rate was similar to cells grown with lactate (0.15 versus 0.13 h^{-1}, respectively). However, pyruvate grown cells did not display a growth lag when exposed to 0.05 mM Cr(VI), and cells had a shorter lag when exposed to 0.1-mM Cr(VI) levels (Fig. 8). These results suggested that pyruvate-grown cells were much less affected by Cr(VI) exposure than lactate-grown cells.

Discussion

D. vulgaris requires hydrogenases and cytochrome c_3 for the reduction of Cr(VI) to Cr(III) (Lovley and Phillips 1994; Chardin et al. 2003; Elias et al. 2004), and Cr(III) can be detected on the cell surface as well as in the periplasmic space (Goulhen et al. 2006). Our results corroborated the association of reduced Cr with cells because culture supernatants from Cr-exposed cells did not inhibit the growth of cells not exposed to Cr(VI). These data indicate that toxic intermediates were not soluble components of the culture supernatant but rather cell associated.

The inhibition of sulfate reduction by other alternative electron acceptors (e.g., U^{6+}, NO_2, Fe^{3+}, Cu^{2+}) has been shown previously (Elias et al. 2004; Chang et al. 2004; He et al. 2006). Microcalorimetric analysis of growing *D. vulgaris* cultures allowed detection of energy production without growth after Cr(VI) exposure (Chardin et al. 2002). However, this study did not measure metabolic substrates or products (e.g., lactate, sulfate, ATP), and Cr(VI) was added directly to growing cells in the presence of high sulfide levels.

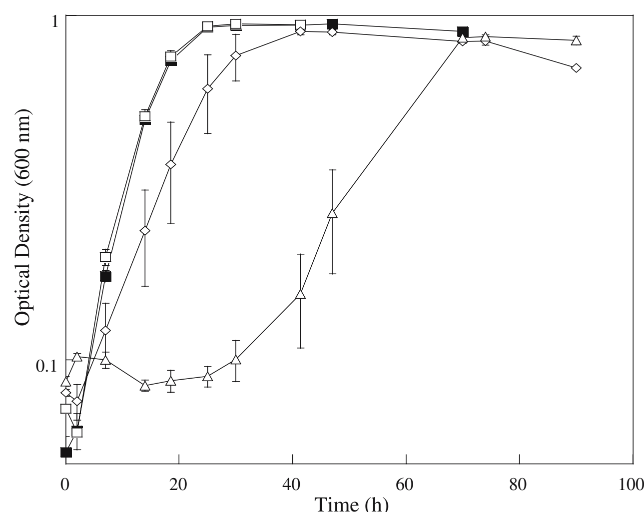

Fig. 8 Washed *D. vulgaris* cells grown with pyruvate as the sole carbon and electron source and exposed to either 0.02 mM (*empty square*), 0.05 mM (*empty diamond*), or 0.10 mM (*empty triangle*) Cr (VI). *Filled square* represents growth on pyruvate alone in the absence of Cr(VI). Growth was monitored by via OD_{600}

Elias et al. (2004) proposed a common pathway for metal and sulfate reduction via cytochrome c_3 that would explain the inhibition of sulfate reduction by U(VI) as both would compete for the same electron carrier. He et al. (2006) showed via global gene expression that nitrite exposure in *D. vulgaris* causes a re-routing of electrons to nitrite away from sulfate for the detoxification of nitrite to ammonia. Our results demonstrated that Cr(VI) exposure decoupled lactate consumption from sulfate reduction, and these results supported the notion that electron flow from cytochrome c_3 is a non-specific process that can be diverted from sulfate by other redox partners including Cr(VI).

Chardin et al. (2002) hypothesized that an increase in ORP during Cr(VI) exposure prevented *D. vulgaris* cells from growing and that the observed energy production without sulfate reduction was used to establish a lower ORP. We did observe longer growth lags upon exposure to higher Cr(VI) concentrations; however, ORP was not significantly altered when different Cr(VI) concentrations were added to the defined medium. When 0.02, 0.05, or 0.10 mM Cr(VI) was added to the medium with washed cells, the ORP was at least −105 mV. These results indicated that an increase in ORP did not prevent the initiation of cell growth nor directly cause the decoupling of carbon oxidation from sulfate reduction. When Cr(VI) was added to pre-reduced medium (e.g., Ti, sulfide, cysteine) at the time of inoculation, a growth lag was still observed. This result indicated that cellular toxicity was not caused directly by the presence of Cr(VI) but rather a by-product of Cr–cell constituent interactions (discussed below) in the studied circumneutral pH ranges.

It is usually assumed that insoluble chromates form when Cr(VI) is reduced to Cr(III); however, soluble complexes can be readily formed in the presence of appropriate substances (e.g., cellular components and metabolites; Pourbaix 1966). In 2002, Puzon et al. showed that a bacterial flavin reductase from *Escherichia coli* could reduce Cr(VI) to Cr(III) and that a soluble Cr(III)–NAD^+ complex was formed (Puzon et al. 2002). Cr(VI) reduction in the presence of organic metabolites was shown to form both soluble and insoluble organo-Cr(III) complexes (Puzon et al. 2005). These studies demonstrated that organo-Cr(III) complexes were readily formed instead of being entirely Cr(III) precipitates.

Recent work with the metal-reducing bacterium, *Shewanella* MR-4, has shown that as Cr(VI) is reduced, growth is hindered and Cr(III) accumulates (Bencheike-Latmani et al. 2007). The authors further showed that a complexing agent

reduced deleterious effects on the cells, and the authors hypothesized that chromium toxicity in *Shewanella* MR-4 was a consequence of Cr(III) either as Cr^{3+}, $Cr(OH)^{2+}$, and/or $Cr(OH)_2$. Soluble Cr(III) has been shown to bind non-specifically with DNA and protein in eukaryotic cells (Vasant et al. 2001; Sugden 2000), and has also been shown to cause lethal double-stranded breaks (Stearns et al. 1995).

Our data suggested that the cells incurred cellular damage via Cr(III), and that energy and/or carbon are consumed to repair the cells before growth can be initiated. The fact that ascorbate and pyruvate can protect the cells from Cr exposure indicated that the cells are susceptible to by-products of Cr(VI) reduction. In addition, the ascorbate or pyruvate has to be present before or during the Cr reduction period. Complexes formed with Cr(III) are currently of interest and may explain how *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 (Puzon et al. 2002, 2005). Mabbett et al. (2002) demonstrated that resting *D. vulgaris* cells could reduce Cr(VI) faster in the presence of ligands, such as citrate, diethylenetriamine pentaacetic acid, and ethylenediamine tetraacetic acid, and that carbonate ion (CO_3^{2-}) could also serve as a complexing agent for Cr(III) that allowed resting cells to reduce Cr(VI).

The fact that pyruvate-grown cells had increased tolerance to Cr exposure compared to lactate-grown cells even though the growth rates were similar also suggested that the ability to complex Cr(III) provides cellular protection. The overall biomass yields for the Cr-exposed cells were slightly lower with pyruvate, and Puzon et al. (2005) recently reported that pyruvate could form complexes with Cr(III) at a slow rate. The presence of pyruvate might provide extra protection once Cr(VI) has been reduced by the cells and may explain the decreased Cr toxicity when cells were grown with this substrate. An additional difference between the two substrates at 0.05 mM Cr(VI) was that lactate-grown cells lagged but pyruvate-grown cells displayed a slower growth rate without a growth lag. In addition, previous studies have shown that pyruvate can yield more energy than lactate during *D. vulgaris* growth (Traore et al. 1981), and more efficient growth might be advantageous for Cr(VI) tolerance. The loss in viability contributed to the growth lag, but the calculated Y_{Lactate} values were not significantly different for the control and the different Cr treatments (3.80±0.08 versus 3.86±0.08 mg protein/mmol lactate, respectively). These data indicated that similar amounts of carbon were oxidized with or without Cr for energy and carbon, and similar results were observed when the acetate to lactate ratio and Y_{Sulfate} were compared (data not shown). For any carbon source, it is unknown if the cells might produce a specific metabolite (e.g., amino acid, TCA intermediate) to complex the Cr(III) or if it is simply a turnover of cellular material adducted with Cr(III) in a non-specific manner (e.g., cytochrome c_3). However, the fate and transport of reduced Cr(VI) should be re-examined under different geochemical conditions and concentrations as well as the possible interactions with biological molecules. Cr(VI)-contaminated sites can differ in geochemical parameters, including carbonate minerals and ions. The cellular responses to Cr exposure need to be evaluated under different biogeochemical conditions.

The described experiments showed that Cr(VI) exposure affected active *D. vulgaris* cells under low sulfide to Cr(VI) ratios by decoupling energy production from carbon oxidation. In addition, organic molecules, such as ascorbate and pyruvate, could alleviate Cr exposure under growth conditions. In natural environments, growth rates are slower and population sizes can be low; therefore, sulfide levels could be low. The characterization of cellular responses to heavy metals at different biomass to metal ratios is crucial to better understand bacterial stress and survival under conditions conducive for heavy metal reduction in situ.

Acknowledgments The research was supported by the United States Department of Energy, Office of Science under the Environmental Remediation Science Program (DOE-ER64125) and the Genomics Program: GTL through contract DE-AC02-05CH11231 between Lawrence Berkeley National Laboratory and the U. S. Department of Energy. In addition, BJG and JDW were supported through the DOE Office of Basic Energy Sciences grant DE-FG02-87ER13713.

References

ATSDR (2004) Toxicological Profile for Chromium; Agency for Toxic Substances and Disease Registry: Atlanta, GA

Bencheike-Latmani R, Obraztsova A, Mackey MR, Ellisman MH, Tebo BM (2007) Toxicity of Cr(III) to *Shewanella* sp. strain MR-4 during Cr(VI) reduction. Environ Sci Technol 41:214–220

Chang IS, Groh JL, Ramsey MM, Ballard JD, Krumholz LR (2004) Differential expression of *Desulfovibrio vulgaris* genes in response to Cu(II) and Hg(II) toxicity. Appl Environ Microbiol 70:1847–1851

Chaplin MF (1986) Monosaccharides. In: Chaplin MF, Kennedy JF (eds) Carbohydrate analysis. IRL, Oxford, pp 1–2

Chardin B, Dolla A, Chaspoul F, Fardeau ML, Gallice P, Bruschi M (2002) Bioremediation of chromate: thermodynamic analysis of the effects of Cr(VI) on sulfate-reducing bacteria. Appl Microbiol Biotechnol 60:352–360

Chardin B, Giudici-Orticoni MT, De Luca G, Guigliarelli B, Bruschi M (2003) Hydrogenases in sulfate-reducing bacteria function as chromium reductase. Appl Microbiol Biotechnol 63:315–321

Cheung KH, Gu JD (2007) Mechanism of hexavalent chromium detoxification by microorganisms and bioremediation application potential: a review. Int Biodeterior Biodegrad 59:8–15

Clark ME, He Q, He Z, Alm EJ, Huang KH, Hazen TC, Arkin AP, Wall JD, Zhou J, Fields MW (2006) Temporal transcriptomic analyses of *Desulfovibrio vulgaris* Hildenborough during electron donor depletion. Appl Environ Microbiol 72:5578–5588

Elias DA, Suflita JM, McInerney MJ, Krumholz LR (2004) Periplasmic cytochrome c3 of *Desulfovibrio vulgaris* is directly involved in H_2-mediated metal but not sulfate reduction. Appl Environ Microbiol 70:413–420

Goulhen F, Gloter A, Guyot F, Bruschi M (2006) Cr(VI) detoxification by *Desulfovibrio vulgaris* strain Hildenborough: microbe–metal interaction studies. Appl Microbiol Biotechnol 71:892–897

He Q, Huang KH, He Z, Alm EJ, Fields MW, Hazen TC, Arkin AK, Wall JD, Zhou J (2006) Energetic consequences of nitrite stress in *Desulfovibrio vulgaris* Hildenborough inferred from global transcriptional analysis. Appl Environ Microbiol 72:4370–4438

Heidelberg JF, Seshadri R, Haveman SA, Hemme CL, Paulsen IT et al (2004) The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. Nat Biotechnol 22:554–559

Iwamoto T, Nasu M (2001) Current bioremediation practice and perspective. J Biosci Bioeng 92:1–8

Lovley DR, Phillips EJ (1994) Reduction of chromate by *Desulfovibrio vulgaris* and its c(3) cytochrome. Appl Environ Microbiol 60:726–728

Mabbett AN, Lloyd JR, Macaskie LE (2002) Effects of complexing agents on the reduction of Cr(VI) by *Desulfovibrio vulgaris* ATCC 29579. Biotechnol Bioeng 79:389–397

Postgate J (1984) The sulfate-reducing bacteria, 2nd edn. Cambridge University Press, Cambridge

Pourbaix M (1966) Atlas of electrochemical equilibria in aqueous solutions. Pergamon, Oxford, UK

Puzon GJ, Petersen JN, Roberts AG, Kramer DM, Xun L (2002) A bacterial flavin reductase system reduces chromate to a soluble chromium(III)–NAD^+ complex. Biochem Biophys Res Comm 294:76–81

Puzon GJ, Roberts AG, Kramer DM, Xun L (2005) Formation of soluble organo-chromium(III) complexes after chromate reduction in the presence of cellular organics. Environ Sci Technol 39:2811–2817

Riley RG and Zachara JM (1992) Chemical contaminants on DOE lands and selection of contaminant mixtures for subsurface science research. DOE/ER-0547T, DE92 014826; http://www.lbl.gov/ERSP/generalinfo/primers_guides/Riley-Zachara1992.pdf

Santos H, Fareleira P, Xavier AV, Chen L, Liu MY, LeGall J (1993) Aerobic metabolism of carbon reserves by the obligate anaerobe *Desulfovibrio gigas*. Biochem Biophys Res Comm 195:551–557

Stearns DM, Kennedy LJ, Courtney KD, Giangrande PH, Phieffer LS, Wetterhahn KE (1995) Reduction of chromium(V1) by ascorbate leads to chromium–DNA binding and DNA strand breaks in vitro. Biochem 34:910–919

Sugden KD (2000) Formation of modified cleavage termini from the reaction of chromium(V) with DNA. J Inorg Biochem 77:177–183

Traore AS, Hatchikian CE, Belaich JP, LeGall J (1981) Microcalorimetric studies of the growth of sulfate-reducing bacteria: energetics of *Desulfovibrio vulgaris* growth. J Bacteriol 145:191–199

Vasant C, Balamurugan K, Rajaram R, Ramasami T (2001) Apoptosis of lymphocytes in the presence of Cr(V) complexes: role in Cr (VI)-induced toxicity. Biochem Biophys Res Commun 285:1354–1360

Viamajala S, Peyton BM, Apel WA, Petersen JN (2002) Chromate/nitrite interactions in *Shewanella oneidensis* MR-1: evidence for multiple hexavalent chromium [Cr(VI)] reduction mechanisms dependent on physiological growth conditions. Biotechnol Bioeng 78:770–778