Microbial reduction of chromium from the hexavalent to divalent state

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microbial; anaerobic; cell surfaces; valence; pathway;
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Abstract

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1. Introduction

Biochemical electron-transport pathways for metal reduction, which are likely the earliest mechanisms of microbial respiration (Vargas et al., 1998) and are utilized by many genera of archaea/eubacteria today, remain poorly understood. Many genera of archaea/eubacteria (e.g., psychrophilic, mesophilic, and thermophilic; Gram positive and negative), common to different soils and sediments, can reduce a wide range of metals including highly-toxic Cr (Cervantes, 1991; Lovley, 1993; Turick et al., 1996; Chen and Hao, 1998; Badar et al., 2000; Fendorf et al., 2000; Kashefi and Lovley, 2000; Wang, 2000; Horton et al., 2006). Chromium is a redox active 3d transition metal with a wide range of possible oxidation states (−2 to +6, in rare cases −4 and −3 are reported) (Cotton and Wilkinson, 1966; Hughes et al., 1979; Sallans et al., 1983; Shupack, 1991; Greenwood and Earnshaw, 1998) of which only two, trivalent Cr(III) and hexavalent Cr(VI), are stable in the majority of terrestrial surface and aqueous environments (Kimbrough et al., 1999). The valence of Cr largely controls the biogeochemical properties of Cr complexes including solubility, adsorption affinity, chemical reactivity, and toxicity. For instance, Cr(VI) species are strong oxidants which act as carcinogens, mutagens, and teratogens in biological systems (Cieglak-Golonka, 1995; Codd et al., 2001; Bagchi et al., 2002; LeVine et al., 2003). The structural similarity of the soluble chromate anion (dominant Cr(VI) species at pH < 6.1) (Brito et al., 1997) to biologically important inorganic anions, such as
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(SO_4^{2-}) and (PO_4^{3-}), is likely responsible for its ability to readily transverse cell membranes, via the sulfate transport system, and be incorporated into cells (see Cervantes et al., 2001; Codd et al., 2001). In contrast, Cr(III) species have low toxicity, in part because their bioavailability is limited by low solubility (Rai et al., 1987, 1989) and the tendency of dissolved Cr(III) to be adsorbed by organic carbon (Fukushima et al., 1995) as well as mineral surfaces (Griffin et al., 1977; Chakir et al., 2002). Mechanisms for Cr(VI) reduction are of particular technological and biological importance because they can convert a toxic, mobile metal into an innocuous, immobile reduced form. Depending on the microbial species, Cr reduction can occur under aerobic and/or anaerobic conditions. Although many bacteria can conserve energy to support growth (i.e., respire) through anaerobic metal (e.g., Fe, Mn) reduction, there are only a few reports that bacteria can conserve energy through anaerobic Cr(VI) reduction (e.g., see Tebo and Obraztsova, 1998) and many of these reports have been challenged (Lovley, 1995). Nevertheless, evidence indicates Cr reduction is an enzymatically catalyzed reaction attributed to soluble proteins for some species or cell membranes for other species (Chen and Hao, 1998). However, little is known about their associated electron-transport pathways. Microbial Cr reduction is a complex process with multiple possible pathways and unstable redox intermediates with intermediate valence between the valences of the stable end members. Evidence of two Cr redox intermediates, Cr(IV) and Cr(V), in the microbial reduction of Cr(VI) have been reported (Suzuki et al., 1992; Myers et al., 2000; Neal et al., 2002; Kalabegishvili et al., 2003). Electron paramagnetic resonance (EPR) measurements, sensitive to non-integer spin transition metals such as Cr(I), Cr(III), and Cr(V), suggested that Cr(V) is a possible redox intermediate for Cr(VI) reduction by Pseudomonas ambigu (G-1) (Suzuki et al., 1992), Arthrobacter oxydans (Kalebighvili et al., 2003), and Shewanella oneidensis (Myers et al., 2000). X-ray photoelectron spectroscopy studies of batch cultures of S. oneidensis with chromate were unable to detect evidence for Cr(V) reduction intermediates, however evidence for Cr(IV) intermediates at cell surfaces was suggested (Neal et al., 2002).

It is widely believed that reduction to Cr(III) is the final pathway step in the microbial Cr(VI) reduction chain because bacterial cells become encrusted with Cr-rich precipitates (Wang et al., 1990; Fude et al., 1994) (see Figs. 1 and 2) and Cr(III) is the only stable, insoluble form. However, this fundamental hypothesis has never been experimentally verified. The assumed termination of the microbial reduction pathways at Cr(III) has important implications to the mechanisms of chromate reduction particularly for Cr(VI)-reducing bacteria with membrane-bound reductases. For example, Cr(VI) reductases are reportedly localized to the cytoplasmic membrane of S. oneidensis (Myers et al., 2000) as well as associated with membranes of Enterobacter cloacae (Wang et al., 1990), Pseudomonas fluorescens (Bopp and Ehrlich, 1988), and Shewanella oneidensis cells from a N_2-bubbled anaerobic culture containing Cr(VI)O_4^{2-} imaged by an ElectronScan E3 environmental scanning electron microscope. Many of the cells exhibit Cr precipitates on their surface; and Cr precipitates not attached to cells are also present. For scale, the cells are several microns in length.
Fig. 3. Valence determination by electron energy loss spectroscopy. (a) Correlation between L-edge fine structure and Cr valence for standards with a range of chemistries. Fine structure was parameterized by the ratio of the integrated intensity of the Cr-L$_3$ edge relative to the Cr-L$_2$ edge (i.e., L$_3$/L$_2$ ratio), and the energy of the Cr-L$_3$ maxima. The Cr(0) and Cr(I) standards plotted are presumed of low-spin configuration. (b) Experimental data from *Shewanella oneidensis* anaerobic cultures containing chromate as the sole terminal electron acceptor with minimal media and lactate as the electron donor: 30 non-encrusted cells incubated in the anaerobic chamber (blue), 16 precipitates encrusting cells from a N$_2$-bubbled culture with no exposure to atmospheric O$_2$ after sectioning (pink), 16 precipitates encrusting cells from a N$_2$-bubbled culture that were exposed to atmospheric O$_2$ after sectioning (orange). The solid data points without error bars represent the mean of the data for a particular Cr standard.

verification that precipitates, which encrust chromate-reducing *S. oneidensis*, are predominantly Cr(III) solids (Dautlon et al., 2002). In this work, the considerably refined EELS technique is applied to study the mechanisms of anaerobic Cr reduction by *S. oneidensis*. Compelling experimental evidence is obtained that demonstrates, unexpectedly, that the generally accepted idea that microbial reduction terminates at Cr(III) is incorrect for, at least, *S. oneidensis*. We demonstrate the terminal microbial reduction step is one that produces Cr(II), and provide evidence that reduction occurs within the cell. These new experimental results place into question the current models...
proposed for Cr(VI) reduction by *S. oneidensis* and perhaps other Cr(VI)-reducing bacteria with membrane-bound Cr reductases.

2. Experimental

2.1. Bacterial cultures

The γ-proteobacterium (Gram-negative), *S. oneidensis* MR-1 (ATCC 700550) (Venkateswaran et al., 1999; Heidelberg et al., 2002), previously classified as *Putrefaciens* MR-1 (MacDonell and Colwell, 1985) and before that as *Alteromonas putrefaciens* (Lee et al., 1977) was isolated from the anaerobic zone of Mn-rich sediments in Oneida Lake, New York (Myers and Nealson, 1988b). *Shewanella oneidensis* is a mesophilic, facultative anaerobe with a remarkably diverse reductive capability that can reduce (in some cases also respire) a variety of terminal electron acceptors, including: O2, V(V), Cr(VI), Fe(III), Mn(IV), Tc(VII), U(VI), nitrite (NO2-), nitrate (NO3-), sulfite (SO42-), thiosulfate (S2O32-), tetraionate (SO32-), furamate, glycine, and trimethylamine-N-oxide (see Myers and Nealson, 1988b; Lovley et al., 1991; Llodio and Macaskie, 1996, Carpentier et al., 2005).

A total of six anaerobic cultures were prepared; two control and four experimental. For each of these cultures, cells of *S. oneidensis* were initially grown under aerobic conditions at 30 °C for 18–24 h in Luria-Bertani (LB) broth (pH 7.0) with continuous agitation. An aliquot (∼5 ml) of the aerobic culture (for one of the controls, cells were first killed by glutaraldehyde and washed) was transferred to anaerobically maintained 125 ml flasks containing ∼50 ml minimal growth medium (adapted from Myers and Nealson, 1988a,b; Lovley et al., 1991; Llodio and Macaskie, 1996, Carpentier et al., 2005).

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2.1.2. Experimental cultures

Four experimental cultures were incubated. In the first, cells were cultured in an anaerobic chamber for 21 days with a total of 1.2 mM of K2Cr(VI)O4 added. Although reduction of Cr(VI) was confirmed by diphenylcarbazide colorimetric assay (see Clesceri et al., 1999), no precipitates were observed in this culture by TEM. Two replicate Cr-reducing cultures incubated for 30 days (total 1.8 mM chromate added) and 97 days (total 4 mM chromate added) also exhibited no precipitate formation. In the fourth culture, cells were incubated with 1 mM K2Cr(VI)O4 using a continuous flow of bubbled N2 (purity 99.9%) for 28 days, and precipitate encrusted cells were observed (Figs. 1 and 2).

2.2. Microcharacterization of individual cells

2.2.1. Specimen preparation for transmission electron microscopy

To preserve the chemical redox state of bacteria and reduction products, all specimens were handled in an anaerobic chamber at each subsequent stage of TEM specimen preparation. Cultures were embedded in resin and in that procedure, an aliquot (1 ml) of each culture was pelleted by centrifugation, and fixed in 5% aqueous solution of glutaraldehyde (1 ml) overnight at room temperature. Fixed cells were washed three times in distilled water, treated with 2% osmium tetroxide (aqueous solution), and again washed three times. The pellets were then processed through a gradual ethanol dehydration sequence, washed in 100% ethanol, and washed in propylene oxide. Increasing concentrations of Spurr’s resin mixed in propylene oxide were infiltrated into the specimen pellet and cured. Embedded specimens were transferred under anaerobic conditions to a second anaerobic chamber (95% N2:5% H2) containing a microtome, and sectioned to ∼70 nm thickness.

For TEM-EELS analysis, a TEM specimen holder was placed in the anaerobic chamber containing the microtome, specimens were mounted in the holder, and the specimen tip of the holder was sealed with an airtight cap. The sealed TEM holder was quickly (<20 s) transferred to a N2-purged glove bag affixed to the microscope goniometer, unsealed under N2-purge, and quickly inserted into the TEM column. Specimens were shipped under anaerobic conditions for scanning (S) TEM-energy dispersive X-ray spectroscopy (EDXS) spectrum imaging analysis.

2.2.2. Microcharacterization by transmission electron microscopy

A JEOL JEM-3010 transmission electron microscope, at the Naval Research Laboratory (NRL), operating at
300 KeV with a LaB₆ electron source was used for EELS analysis. The EELS spectrometer (GIF200) was calibrated to 855.00 ± 0.02 eV for the energy of the Ni–L₃ edge maxima of NiO. Spectra were collected in TEM diffraction mode by the spectrometer using an illumination angle 2α = 4–10 mrad, a collection angle of 2β = 11.2 ± 0.3 mrad, a 2-mm diameter entrance aperture, and an energy dispersion of 0.1 eV/channel. “Summed” spectra of the O–K/Cr–L core-loss regime were acquired with an integration time of 2 s per spectrum (100 spectra summed) or 4 s per spectrum (40 spectra summed) for precipitates, and 4 s per spectrum (200 spectra summed) for bacteria. The mean energy drift (regardless of sign/direction) of the spectrometer during acquisition of core-loss spectra was 0.18 eV for precipitates and 0.23 eV for cells. Six summed core-loss spectra (along with accompanying zero-loss spectra) were collected for each bacterium (at different locations within the cell) or each precipitate; results were averaged and reported with the statistical standard error. Full details of the methodology of the EELS measurements and the EELS technique for Cr-valence determination have been described previously (Daulton and Little, 2006). The following standards were used to determine partial cross sections for elemental quantification by EELS: CrO₂, Cr₂O₃, LaCrO₃, NdCrO₃, and NdCrO₄. Since EELS spectra were preferentially collected from regions inside the cell with the highest Cr signal, reported cellular Cr concentrations represent an upper limit for mean cell concentrations, or a lower limit for localized concentrations, within the cell. A Hitachi HD-2000 dedicated scanning transmission electron microscope, at Oak Ridge National Laboratory (ORNL), operating at 200 keV with a cold-field emission electron source was used to acquire Cr elemental maps using spectrum-imaging, EDXS techniques. This instrument was equipped with a secondary electron detector, a bright-field detector, a high-angle annular dark-field (HA-ADF) detector, and a Noran 40 mm² solid-state X-ray detector with a collection solid angle of 0.3 sr relative to the specimen. Elemental EDXS maps were collected at 256 x 192 pixel resolution over a period corresponding to 1.4 x 10⁴ s of livetime.

3. Results

Two anaerobic control cultures were examined by TEM: glutaraldehyde-killed S. oneidensis with chromate as the sole terminal electron acceptor and viable S. oneidensis with Cr(III) as the only available terminal electron acceptor. Two series of experimental anaerobic cultures of viable S. oneidensis with chromate as the sole terminal electron acceptor were examined: one cultured in an anaerobic chamber and one using a continuous flow of bubbled N₂. Lack of Cr(VI) reduction in the glutaraldehyde-killed S. oneidensis control and Cr(VI) reduction in the experimental cultures were confirmed by diphenylcarbazide colorimetric assay (see Clesceri et al., 1999). Further, in earlier studies, no Cr(VI) reduction was observed under aerobic or anaerobic conditions in cultures of heat-killed S. oneidensis cells (see Lowe et al., 2003). Intracellular regions (~100–200 nm diameter) of cross-sectioned cells that sometimes included parts of the cell wall were analyzed by TEM–EELS. Bacteria exhibit a range of geometric cross sections in TEM thin sections of embedded cultures, and in some of these cross sections Cr precipitates associated with their surface can contribute to the EELS Cr–L edge from the cell. Therefore, only cells lacking Cr precipitates associated with their surface were examined by EELS. The EELS spectra demonstrated no significant Cr-adsorption edges present in 30 cells from either of our control cultures, in contrast to our experimental cultures where many bacteria exhibited Cr adsorption edges. Based on these observations, Cr(VI) reduction is attributed to live cells.

Three different anaerobic-chamber cultures exhibited no Cr precipitates encrusting cells or separate from cells. Precipitate formation was only observed in the N₂-bubbled culture, suggesting that precipitate formation in the N₂-bubbled culture is associated with an oxidant that was far less abundant in the anaerobic chamber culture. Since the same growth media was used in all cultures, trace oxidants in the growth media can be ruled out. Hexavalent Cr can also be ruled out since the N₂-bubbled culture had less total Cr exposure than the anaerobic chamber cultures. The only other possible oxidant that can differ in exposure levels between the N₂-bubbled culture and the anaerobic chamber culture is oxygen. Since competing oxygen scavengers are present in the cultures under conditions where the associated reactions are oxygen limited, the oxygen input into the cultures is more relevant than the steady state oxygen levels achieved. In this regard, the purity of the nitrogen used in the N₂-bubbled culture and in anaerobic chamber was 99.9% and 99.999%, respectively. Furthermore, the anaerobic chamber had an active Pd catalyst that removed oxygen from the gas in the chamber. Therefore, the N₂-bubbled culture was exposed to at least two orders of magnitude more oxygen than the cultures in the anaerobic chamber.

As mentioned previously, Cr precipitates associated with cell surfaces can contribute to the Cr–EELS edge from the cell. Therefore, valence of intracellular Cr was determined for only cells from the anaerobic chamber cultures because they lacked precipitates encrusting their surface. The EELS spectra from these cells exhibit Cr L₃ peak maxima that are significantly lower than that of Cr(VI) and Cr(V) species (Fig. 3b), clearly indicating no appreciable bioaccumulation in the cells of the toxic, highly-oxidized species. In the EELS/valence correlation plot (Fig. 3), part of the low-spin Cr(II) region overlaps with part of the Cr(III) region. Nevertheless, nearly all of the cell spectra (87%) are within experimental error of the low-spin Cr(II) region characteristic of many organometallic Cr(II) compounds (Daulton and Little, 2006) while in contrast 57% are within experimental error of the Cr(III) region (Fig. 3b). Of the 13% of the cell spectra that lie outside experimental error of the low-spin Cr(II) region, only one
Divalent Cr anions can readily bind in the net negatively charged extracellular biopolymers, and these biopolymers permeate the surfaces of Cr(III) precipitates. The greatest measurable effect that would be observed in Fig. 3 from contributions of low-spin Cr(II) to the EELS spectra of Cr(III) precipitates, where Cr(II) < Cr(III) concentrations, is a shift in relative peak heights (i.e., an increase in the L$_3$/L$_2$ ratio) from that expected for Cr(III) accompanied by a relatively small shift (i.e., decrease) in the position of the L$_3$ peak. This is in qualitative agreement with our observations. The EELS measurements provide compelling evidence that the bacterial cells contain Cr predominantly in the divalent form with no appreciable bioaccumulation of toxic, highly-oxidized Cr.

The Cr/O ratio for *S. oneidensis* cells examined by EELS was quantified from the simultaneously collected O-K and Cr-L edges using partial cross sections determined from the Cr standards. Cellular Cr concentration was estimated from the measured Cr/O ratio by assuming C$_{160}$(H$_{280}$O$_{80}$)N$_{30}$P$_{2}$S as the mean cell composition (McKinley and Grogan, 1991), which provides a ratio of oxygen atoms to the total atoms in the biomass of the cell. Estimated concentrations are relatively high at between 0.03 and 0.09 g Cr/g bacterium (equivalent to 0.5–1.2 at.% Cr) for those cells measured by EELS (Fig. 4). Amorphous precipitates that encrust cells are generally assumed to be hydrated chromium hydroxide, Cr(OH)$_3$·nH$_2$O, and EELS-measured Cr/O ratios of precipitates are between 0.06 and 0.09. In comparison, EELS partial cross sections calculated from the hydrogenic (with white line corrections) and Hartree–Slater models yielded Cr/O ratios and Cr concentrations that were larger than those determined from Cr standards (as reported here) by a factor of 1.18 and 1.21, respectively.

Elemental EDXS maps of non-encrusted (fixed, embedded, and cross-sectioned) *S. oneidensis* cells from anaerobic cultures demonstrate Cr, identified as divalent by EELS, concentrated near the cell wall and extending into the cytoplasm (Fig. 5). In particular, the STEM HA-ADF image of Fig. 5a displays two dark bands along the perimeter of the bacteria that are separated by an ~11 nm thick low-contrast band. These bands are consistent with the Gram-negative cell wall structure of an outer membrane and cytoplasmic membrane separated by the periplasmic space. As best illustrated in the lower left of Fig. 5d (see also Fig. 6), intracellular Cr appears more concentrated along the inner most band (cytoplasmic membrane) than the outer most band (outer membrane and possibly also the peptidoglycan layer; e.g., see Hobot et al., 1984; Beveridge, 1999).

In comparison to our TEM–EELS/STEM–EDXS techniques, valence and distribution of Cr associated with the Cr-reducing bacteria *P. fluorescens* have been studied using synchrotron, high-energy X-ray fluorescence (XRF) (Kemner et al., 2004). Similar to *S. oneidensis*, the Cr(VI) reducers of *P. fluorescens* are reportedly associated with cellular membranes (Bopp and Ehrlich, 1988). Although synchrotron XRF elemental maps of whole cells suggest Cr uptake by aerobically cultured *P. fluorescens* exposed...
Fig. 5. Spatial distribution of intracellular Cr within a cross-sectioned (=70 nm thick slice) *Shewanella oneidensis* cell from an anaerobic chamber culture containing Cr(VI)O_4^{2-}. (a) Scanning (S) transmission electron microscopy (TEM) high-angle annular dark-field (HA-ADF) image in reversed contrast. Energy dispersive X-ray spectrum STEM images of (b), Os, and (c), Cr. (d) Superposition of the images (a) and (c). All images are projections through the cross section of the cell, and the sharp contrast of the two bands near the perimeter of the cell suggests a principle ellipsoidal axis of the bacteria lies nearly parallel and in proximity to the plane of the cross section. Small (<10 nm) discrete specks of dark contrast in the STEM HA-ADF image are Os-rich precipitates that are TEM specimen preparation artifacts from osmium tetroxide used to stain the cells. Scale bar is 100 nm.

4. Discussion

Our EELS data suggest that at least one-third, if not most, of the cells of *S. oneidensis* analyzed from Cr(VI)-reducing anaerobic-chamber cultures contain Cr predominantly in the divalent state. Given that these cells did not have precipitates encrusting their surface, this suggests that Cr(II) accumulation precedes formation of Cr(III) precipitates on cell surfaces. This is further supported by the precipitate forming N_2-bubbled cultures via the EELS detection of Cr in cells that have not yet formed precipitates on their surface. To further evaluate if the intracellular Cr(II) observed was the product of reduction of Cr(III) precipitates that may have formed first on bacterial cells, a control using Cr(III)PO_4·nH_2O as the only potential electron acceptor was examined. Since precipitates from a Cr(VI) microbial culture are not easily isolated from the cells, it is not possible to use them in the control. Precipitates are generally identified as Cr(OH)_3·nH_2O, although their mineral chemistry has not been rigorously characterized. Similar to Cr(OH)_3·nH_2O, CrPO_4·nH_2O is sparingly soluble and dissociates in water to form Cr(III) cations. This is relevant because it has been reported that the uptake of Cr(III) by *Escherichia coli* can vary for different aqueous Cr(III)-ion species (Plaper et al., 2002). Since the
solubility products, $K_{sp}$ of Cr(OH)$_3$ and CrPO$_4$ are 6.7 x 10$^{-31}$ and 2.4 x 10$^{-23}$, respectively, the latter may provide greater Cr(III) bioavailability and a better test for possible Cr(III) reduction. Additionally, the anion of CrPO$_4$ dissolution, phosphate, is a micronutrient for bacteria that is required as a component of ATP, nucleic acids as well as such enzymes as NAD, NADP, and flavins. The lack of cellular accumulation of Cr(II) in the control culture where Cr(III) was the only available terminal electron acceptor supports the inference that Cr(II) is not a product of reductive dissolution of Cr(III) precipitates that once encrusted those cells. Further, it is known that Cr(VI) can readily transverse cell membranes via the sulfate transport system and be incorporated into cells (see Cervantes et al., 2001; Codd et al., 2001). Our TEM-EELS/STEM-EDXS results demonstrate that Cr(II) is concentrated near the cytoplasmic membrane and extending into the cytoplasm of the S. oneidensis cells, consistent with the reported localization of Cr(VI) reductases (Myers et al., 2000). Therefore, our observations suggest that reduction to Cr(II), not Cr(III), is the final microbial pathway step in the reduction of Cr(VI) for S. oneidensis under anaerobic conditions. Further, these points suggest that the final microbial pathway is intracellularly localized.

If the final microbial pathway is intracellularly localized, then removal of intracellular reduction products is critical to prevent accumulation of these products within the cell or cell-wall structure. Otherwise, unless the Cr-reduction capacity of the cells was limited, these products would accumulate to levels sufficiently high to detrimentally impact cellular processes. Therefore, termination of the microbial reduction sequence at Cr(II) rather than at Cr(III) has a large biological advantage for bacteria with intracellularly localized Cr reductases. This is because Cr(II) is soluble and can be readily expelled from the cell. In contrast, Cr(III) exhibits solubility above 10 µM concentration only under very acidic (pH ≤ 5) or very basic (14 ≤ pH) conditions (Rai et al., 1987, 1989) and, although Cr(III) can be solubilized by complexation, most soluble organo-Cr(III) complexes observed are >6 kDa (Puzon et al., 2005) and because of their size are not easily transported across Gram-negative outer membranes. The exclusion limit for Gram-negative outer membranes is ~1.5 kDa as suggested by vancomycin, a large (1.5 kDa) antimicrobial agent effective against Gram-positive bacteria but inactive against a large majority of Gram-negative bacteria because it cannot cross their outer membrane (see von Graevenitz and Bucher, 1983; Arthi et al., 2003; Yang et al., 2006).

Once expelled from the cell, many Cr(II) ions would bind in the negatively-charged extracellular biopolymer surrounding the cell. Removed from cell reductases, Cr(II) would oxidize in the presence of oxidizers to the more stable trivalent state forming Cr(III) organo-complexes and/or precipitated Cr(III) hydroxides. In otherwise nearly identical incubations, Cr(III) precipitate formation was observed in N$_2$-bubbled cultures but not in three replicate anaerobic-chamber cultures. This suggests that trace oxygen in the N$_2$-bubbled culture may be necessary for the growth of Cr(III) precipitates. Precipitates that were not attached to any cells were also observed in N$_2$-bubbled cultures. Many of these precipitates may have initially formed on, and were later detached from, cell surfaces.

5. Summary and conclusions

We demonstrate that the widely accepted hypothesis that the final microbial pathway for Cr(VI) reduction is one that produces Cr(III) is incorrect. Using newly developed TEM-EELS valence determination techniques, we demonstrate that S. oneidensis reduced Cr(VI) to Cr(II) in anaerobic cultures where chromate was the sole terminal electron acceptor. We further show that the Cr(II) is concentrated, at relatively high levels of ~0.03–0.09 g Cr/g bacterium, near the cytoplasmic membrane and extending into the cytoplasm of S. oneidensis cells, consistent with the reported localization of Cr(VI) reductases (Myers et al., 2000). These observations suggest the final microbial pathway for Cr(VI) reduction is intracellularly localized. Our results provide important experimental data that is necessary for the development of accurate electron-transfer bioproduction models and will eventually lead to a detailed understanding of microbial Cr(VI) reduction. Further, these results represent the first compelling evidence for the formation of Cr(II) in biological systems (e.g., see Levin and Lay, 2005) and may be relevant to the wider scope of understanding the poorly known physiological and cytotoxic mechanisms of Cr in biological systems (e.g., see Vincent, 2000; Bagchi et al., 2002; Levin et al., 2003).

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