

1     **The response of *Shewanella oneidensis* MR-1 to Cr(III) toxicity**  
2                                   **differs from that to Cr(VI)**

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16  
17     **ABSTRACT**

18  
19     Chromium is a contaminant of concern that is found in drinking water in its soluble,  
20     hexavalent form [Cr(VI)] and that is known to be toxic to eukaryotes and prokaryotes.  
21     Trivalent chromium [Cr(III)] is thought to be largely harmless due to its low solubility and  
22     inability to enter cells. Previous work has suggested that Cr(III) may also be toxic to  
23     microorganisms but the mechanism remained elusive. In this work, we probe the toxicity  
24     of Cr(III) to *Shewanella oneidensis* MR-1, a bacterium able to reduce Cr(VI) to Cr(III) and  
25     compare it to Cr(VI) toxicity. We found evidence for Cr(III) toxicity both under Cr(VI)  
26     reducing conditions, during which Cr(III) was generated by the reduction process, and  
27     under non-reducing conditions, when Cr(III) was amended exogenously. Interestingly,  
28     cells exposed to Cr(III) (200  $\mu$ M) experienced rapid viability loss as measured by colony-  
29     forming units on Luria-Bertani (LB) agar plates. In contrast, they maintained some  
30     enzymatic activity and cellular integrity. Cr(VI)-exposed cells exhibited loss of enzymatic  
31     activity and cell lysis. The loss of viability of Cr(III)-exposed cells was not due to  
32     membrane damage or to enzymatic inhibition but rather appeared to be associated with an  
33     abnormal morphology that consisted of chains of membrane-enclosed units of irregular  
34     size. Exposure of abnormal cells to growth conditions resulted in membrane damage and  
35     cell death, which is consistent with the observed viability loss on LB plates. While Cr(VI)  
36     was taken up intracellularly and caused cell lysis, the toxic effect of Cr(III) appeared to be  
37     associated with extracellular interactions leading to an ultimately lethal cell morphology.

## 1. INTRODUCTION

Hexavalent chromium [Cr(VI)], usually as the chromate anion, has historically been released into ground and surface waters by diverse industrial processes (ATSDR, 2004) and has been designated a pollutant of concern. Chromate is freely soluble in water, has well-studied toxic effects on both eukaryotes and prokaryotes (Alcedo and Wetterhahn, 1990; EPA, 1998; Cervantes et al., 2001), and can enter many cell types via sulfate or other active transporters (Ramírez-Díaz et al., 2008). In contrast, trivalent chromium [Cr(III)] is not efficiently transported into most cells (Ramírez-Díaz et al., 2008) and forms hydroxide or phosphate precipitates that are immobile in many aqueous systems at neutral pH (Remoundaki et al., 2007). Because of the low toxicity, insolubility and environmental immobility of Cr(III), microbial reduction of soluble Cr(VI) to create Cr(III) minerals has been suggested as a means to decrease the toxicity and the transport of chromium in contaminated sites (Palmer and Wittbrodt, 1991).

A potential limitation of microbial bioremediation is that the Cr(VI) reduction process seems to be self-poisoning in model organisms (Middleton et al., 2003; Viamajala et al., 2003; Bencheikh-Latmani et al., 2007). For example, the bacteria *Shewanella oneidensis* MR-1 and *Shewanella* sp. strain MR-4, which rapidly reduce Cr(VI) and initially metabolize normally in the presence of 100-200  $\mu$ M Cr(VI) as chromate, were found to gradually lose this ability and to become less viable as the Cr(III) reduction product appeared (Bencheikh-Latmani et al., 2007; Gorby et al., 2008). This toxic effect could be mimicked by the addition of freshly-prepared CrCl<sub>3</sub>, which yielded low  $\mu$ M concentrations of transiently soluble Cr(III) species because of slow precipitation kinetics. In contrast, no effect was observed after the addition of fully-precipitated Cr(III) from aged solutions or when Cr(III)-complexing ligands were added to decrease the availability of Cr(III). These data indicate a toxicity of transiently soluble Cr(III) species (Bencheikh-Latmani et al., 2007). Other support for this idea comes from kinetics of Cr(VI) metabolism by strain MR-1, for which modeling suggests two processes that reductively remove Cr(VI) from solution, one that is inhibited by its product [ordinarily Cr(III)] and one that is not (Viamajala et al., 2003). In addition, the existence of reactive and potentially toxic Cr(II), Cr(IV) and Cr(V) intermediates or products has also been suggested for Cr(VI) reduction by *S. oneidensis* MR-1 (Daulton et al., 2007), mainly during experiments that lasted for several weeks.

*Shewanella oneidensis* MR-1 is a Gammaproteobacterium that is capable of dissimilatory reduction of a wide range of metals, minerals, and some organic compounds (Beliaev et al., 2005; Kolker et al., 2005; Bretschger et al., 2007). Reduction and recovery of metals from iron- and manganese-containing minerals by strain MR-1 can involve nanowires (Gorby et al., 2006; El-Naggar et al., 2010) and perhaps also membrane vesicles (Gorby et al., 2008). Genomic analysis indicates that *S. oneidensis* may produce up to 42 different cytochromes (Meyer et al., 2004), many of which are localized in the outer membrane (Myers and Myers, 2002; Kolker et al., 2005; Bretschger et al., 2007; Shi et al., 2008). Among the latter are the *c*-type decaheme cytochromes MtrC and OmcA, which occur in a complex (Shi et al., 2006) that is required both for the reduction of many metals (Beliaev and Saffarini, 1998; Beliaev et al., 2001). Mutation of MtrC (or of the related proteins MtrA, MtrB, CymA, alone or in combination) yields a partial decrease in the

1 reduction of Cr(VI) or U(VI), but not complete inhibition, suggesting the existence of  
2 multiple Cr(VI) and U(VI) reduction pathways (Bencheikh-Latmani et al., 2005), which is  
3 consistent with the existence in the *S. oneidensis* MR-1 genome of numerous MtrABC  
4 paralogs (Coursolle and Gralnick, 2010). In contrast, mutation in *ccmC*, which is required  
5 for the maturation of *c*-type cytochromes, produces a phenotype without any detectable *c*-  
6 cytochromes (Bouhenni et al., 2005) and completely inhibits the reduction of U(VI)  
7 (Marshall et al., 2006). Here, we test the abovementioned mutant to establish its  
8 phenotype with respect to Cr(VI) reduction. If unable to reduce Cr(VI), this mutant can be  
9 used to distinguish the toxic effects of Cr(VI) from those of Cr(III) because Cr(VI)  
10 exposure can be studied in the absence of the Cr(III) that the wild type might otherwise  
11 produce by reduction.  
12

13 The goal of this paper is to explore the cytotoxic effects on strain MR-1 of the  
14 Cr(III) reduction product, in comparison to those of Cr(VI) in chromate. We first confirm  
15 that Cr(VI) reduction in this strain is indeed self inhibitory, that Cr(III) added as freshly-  
16 dissolved CrCl<sub>3</sub> is also toxic, and that CrCl<sub>3</sub> is more toxic at pH 6, at which inorganic  
17 Cr(III) species are more soluble than at pH 7.2 (Rai et al., 2004; Remoundaki et al., 2007).  
18 We then demonstrate that the toxic effects of CrCl<sub>3</sub> on strain MR-1 differ markedly from  
19 those of Cr(VI). In particular, CrCl<sub>3</sub> exposure produces a characteristic pathology typified  
20 by the appearance of small, irregularly placed membrane-enclosed units (probably either  
21 membrane vesicles or other products of abnormal cell division) that initially are still able  
22 to take up and retain vital dyes, but are associated with cells that cannot form colonies  
23 when plated on LB agar.  
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## 26 **2. MATERIALS AND METHODS**

### 27 **2.0. Cultures, media, and general methods.**

28 **2.0.1. Cultures and media.** *Shewanella oneidensis* MR-1, isolated by Myers and Nealson  
29 (1988) from anoxic sediments of Lake Oneida, NY, USA, was obtained from Oak Ridge  
30 National Laboratory, USA. *S. oneidensis* MR-1 *ccmC* mutant, which was originally  
31 designated mutant BG148 (Bouhenni et al., 2005), was kindly provided by D. Saffarini  
32 and was always grown in media supplemented with  $\geq 100$   $\mu$ g of kanamycin/ml, with  
33 periodic restreaking to prevent the accumulation of revertants. Cultures stored at -80° C  
34 were streaked on Luria-Bertani (LB) agar and maintained in LB broth or SM medium  
35 (Supplementary Table 1) (Bencheikh-Latmani et al., 2007). To avoid complexation of  
36 Cr(III), most experiments involving Cr used a maintenance medium (MM), which  
37 contained in grams per liter (Supplementary Table 1): NaCl, 0.68; KCl, 0.3; MgCl<sub>2</sub>\*6H<sub>2</sub>O,  
38 0.285; Na<sub>2</sub>SO<sub>4</sub>, 0.3975; NH<sub>4</sub>Cl, 0.15; Na<sub>2</sub>HPO<sub>4</sub>, 0.0125; CaCl<sub>2</sub>, 0.0056; D(+) galactose  
39 (Fluka), 20 g; and either HEPES (4-(2-hydroxyethyl)-1-Piperazineethanesulfonic acid) or  
40 MES (2-(N-morpholino)ethane sulfonic acid) buffer, 40 g. The pH was adjusted to 7.2  
41 (HEPES buffer) or 6 (MES buffer) with NaOH. MM maintained the viability of *S.*  
42 *oneidensis*, but provided only very slow growth with protein doubling times of 10-15  
43 hours at culture OD<sub>600</sub> readings of  $\geq 0.1$ . High salts medium (HSM) was isotonic MM  
44 supplemented with 8 g of NaCl per liter (Supplementary Table 1). HSM was utilized to  
45 prevent osmotic stress in cultures during dilution from LB broth, which contains 10 g of  
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47

1 NaCl per liter. Unless otherwise stated, all chemicals were ultrapure or reagent grade from  
2 Sigma-Aldrich. MilliQ water was used for solutions.

3  
4 **2.0.2. Optical density, viable counts and microscopic cell counts.** The optical density at  
5 600 nm ( $OD_{600}$ ) was measured with plastic 1 ml cuvettes in an Eppendorf BioPhotometer.  
6 To determine the viable colony forming units / ml (cfu/ml), 50 or 100  $\mu$ L samples of  
7 cultures were appropriately diluted in HSM or MM and spread (25  $\mu$ L, 100 $\mu$ L, and 250  
8  $\mu$ L) onto LB agar, which was incubated at 25° or 30° C and then counted for bacterial  
9 colonies. Standard deviations were calculated in Excel, ordinarily from 3 plates. For  
10 microscopic counts of total cells (live plus dead), 10-50  $\mu$ l samples (depending on cell  
11 density) were added to 1 ml of a 15  $\mu$ g/ml solution of H3332 (Hoechst 3332; bisbenzimidazole  
12 H33342, AppliChem BioChemica) in phosphate-buffered saline (PBS) pH 7.2, reacted for  
13 20-30 minutes, slowly filtered onto a 25 mm diameter black filter (polycarbonate (PC, 0.2  
14  $\mu$ m pore size, Sterlitech PCTB0225100) or mixed cellulose ester (MCE, 0.45  $\mu$ m pore  
15 size, Membrane Solution and BGB Analytik)), observed for fluorescence on a Nikon  
16 Eclipse E800 microscope with excitation at 350 nm and emission at 461 nm, and  
17 photographed. The number of cells in 3-10 photomicrographs of each sample was counted  
18 visually or automatically with a Matlab computer program. Standard deviations among  
19 the field views, calculated with Excel or Matlab, were less than 16% of each mean, with an  
20 overall average of 7% of the mean. Since cells clumped near the pores of PC filters,  
21 complicating automated counting, most experiments used MCE filters.

22  
23 **2.0.3. DNA and protein assays.** Duplicate or triplicate 1 ml samples were centrifuged  
24 for 4 minutes at 16,000 x g in 1.5 ml microfuge tubes and the supernates were carefully  
25 removed without disturbing the pellets. Each cell pellet was frozen at -20° C for at least 1  
26 day, thawed at room temperature, resuspended in 0.015% Triton X-100, mixed vigorously  
27 on a Vortex mixer, heated at 95° C for 15 minutes, iced, vortexed vigorously and  
28 centrifuged at 16,000 x g for 4 minutes at 4° C to remove particulates. Each supernatant  
29 fluid was transferred to an autoclaved (DNase-free) 1.5 ml microfuge tube, stored at 4° C  
30 and used within 24 hours for both DNA and protein assays. For DNA, 5 or 10  $\mu$ l of each  
31 sample were added (in duplicate) to 190  $\mu$ L of 200-fold-diluted Quant-iT™ dsDNA HS  
32 reagent (Invitrogen). Readings in a QUBIT spectrophotometer (Invitrogen) were  
33 compared to those of Quant-iT™ dsDNA HS standards #1 and #2. For protein assays by  
34 the Bradford method (Bradford, 1976), 80  $\mu$ L portions of each sample was mixed (in  
35 duplicate) with 720  $\mu$ L of MilliQ water and 200  $\mu$ L of Bradford reagent (BioRad).  
36 Absorbance at 595 nm was read after 5, but before 60, minutes of reaction and analyzed in  
37 comparison to standards containing 0, 2, 4, 6, 8, and 10  $\mu$ M bovine serum albumin (BSA)  
38 in 800  $\mu$ L of 0.0015% Triton X-100 and 200  $\mu$ L of Bradford reagent.

39  
40 **2.0.4. Cr(VI) assay.** Duplicate or triplicate 1 ml samples were centrifuged for 4 minutes  
41 at 16,000 x g in 1.5 ml microfuge tubes. The supernates were removed, filtered through  
42 0.22  $\mu$ m pore-size MCE syringe filters (Fisher), and assayed for Cr(VI) by the  
43 diphenylcarbazide (DPC) method (Urone, 1955) or for total Cr by ICP-OES (PerkinElmer  
44 Plasma 2000).

45  
46 **2.0.5. Triple staining to differentiate live and dead cells.** Stock solutions were (per ml):  
47 1 mg of propidium iodide (PI, Fluka) in MilliQ water, 3 mg of H33342 (Hoechst 33342,

1 bisbenzimidazole H33342, AppliChem BioChemica) in MilliQ water, and 6 mg of 5(6)-  
2 carboxyfluorescein diacetate (CFDA, Sigma) in 1 ml of DMSO. The staining solution  
3 contained: 5 µg of PI, 15 µg of H33342, and 30 µg of CFDA per ml of phosphate buffered  
4 saline (PBS), pH 7.4. A 10 to 50 µl portion of each culture (see details of individual  
5 experiments) was added to 1ml of staining solution and reacted in the dark for 20-30  
6 minutes. Microscopic observation was done in wet mounts (2.5 µl of sample covered by a  
7 22x22 mm cover slip) because the percentage of PI-stained cells increased after the  
8 hydrodynamic stress of filtration onto PC membranes. Each mount was examined with a  
9 Nikon Eclipse E800 microscope equipped with Nikon UV-1A, B-2A and DIA-ILL filters.  
10 Multiple visual fields (usually 3-10) of each preparation at each setting were photographed  
11 (80 ms exposure; 2.4x gain) and analyzed with a Matlab program written by P. Borer.  
12 This program automatically counted the number of cells of each fluorescence type (red for  
13 PI, green for CFDA, blue for H33342) and calculated the standard deviation of the counts  
14 among 3-10 field views.

15  
16 **2.0.6 Formaldehyde (paraformaldehyde) fixation of cultures.** Fresh formaldehyde was  
17 prepared from paraformaldehyde (Kiernan, 2000). Aliquots of the same cultures that were  
18 used in each experiment were centrifuged at 10,000 x g for 5 minutes, resuspended in 4%  
19 formaldehyde, reacted for 30 minutes at room temperature, washed by four cycles of  
20 centrifugation (10,000 x g) and resuspension in MM medium salts, and finally suspended  
21 in MM medium. Triplicate or quadruplicate 1 or 2 ml samples were taken for DNA and  
22 protein assays.

23  
24 **2.1. Cr(VI) reduction kinetics for wild type *S. oneidensis* MR-1 and its *ccmC* mutant.**

25 Two experiments were done on the same day with the same materials and experimental  
26 conditions, but with inoculum cultures that had been grown in different media: SM lactate  
27 medium (SML), pH 7.2, for experiment 1 and LB medium for experiment 2. Both  
28 inoculum cultures were grown to an OD<sub>600</sub> of 1, centrifuged, and resuspended in SM  
29 lactate, pH 7.2. The protein concentration after resuspension was 8 µg/ml, which is close  
30 to OD<sub>600</sub> = 0.1 for these strains. Protein was thought to be a more reliable measure of  
31 enzymatic content than OD<sub>600</sub> because the *ccmC* mutant has a slightly different  
32 protein/OD<sub>600</sub> ratio than the wild type. As a control, a portion of the SM lactate culture of  
33 each organism was also killed and fixed with 4% formaldehyde (freshly-prepared from  
34 paraformaldehyde), washed six times to remove residual formaldehyde, and resuspended  
35 to the same protein concentration as the living cultures. All resuspended cell suspensions  
36 were then diluted 1/10 into anaerobically-prepared 100 ml serum bottles filled with 50 ml  
37 of SM lactate, pH 7.2, containing 111 µM Na<sub>2</sub>CrO<sub>4</sub> (100 µM after the addition of cells).  
38 The bottles were incubated at 30° C and shaken at 140 rpm. At timed intervals, 1 ml  
39 samples were removed with a syringe, centrifuged, filtered and assayed for Cr(VI) by the  
40 DPC method (Urone, 1955).

41  
42 **2.2. Effects of pH and Cr(VI) reduction on Cr(VI) toxicity.** Cultures of wild type MR-  
43 1 and the *ccmC* mutant were grown overnight in SM, washed twice by centrifugation at  
44 4,000 rpm during 10 min at 4° C and resuspended in MM, pH 6 or pH 7.2. Portions (11  
45 ml) of each organism at each pH were then transferred with a 21-gauge needle to a set of  
46 anaerobically-prepared 200-ml serum bottles containing 100 ml of MM at the appropriate  
47 pH. One ml samples were removed with a syringe, diluted appropriately in MM, and

1 plated onto LB agar. Immediately thereafter, half of the bottles were supplemented with  
2  $\text{Na}_2\text{CrO}_4$  to a final concentration of 100  $\mu\text{M}$ . At timed intervals, samples were removed  
3 for viable counting and for DPC assays of Cr(VI) in solution.  
4

5 **2.3. Comparison of Cr(III) and Cr(VI) effects at non-reducing conditions.** Wild type  
6 MR-1 and the *ccmC* mutant were grown to an  $\text{OD}_{600}$  of 0.3 in LB broth and then  
7 centrifuged at 2,080 x g during 10 min at 4° C. Pellets were resuspended in HSM, pH 6,  
8 and diluted to an  $\text{OD}_{600}$  of 0.5 into a set of 50 ml Falcon tubes each containing 10 ml of  
9 HSM, pH 6. Various tubes contained 0, 30, or 200  $\mu\text{M}$   $\text{CrCl}_3$  or  $\text{Na}_2\text{CrO}_4$ . The tubes were  
10 left loosely capped at room temperature in an inclined position to enhance oxygen  
11 diffusion. Samples were taken at 0, 6 and 24 hours for viable counting. Other samples  
12 were taken at 0, 1, 3, 6 and 22 hours for staining and Cr assays. Triple-stained  
13 preparations were observed in wet mounts. H33342-stained preparations were also filtered  
14 onto black polycarbonate filters and used for total cell counts.  
15

16 **2.4. Cellular uptake of radio-labeled  $\text{CrCl}_3$  or chromate.** *S. oneidensis* MR-1 wild type  
17 cells from a 27-hour culture in SM with lactate, pH 6, were centrifuged at 4,500 x g for 5  
18 min. Half of the cell pellet was resuspended in MM, pH 6, to an  $\text{OD}_{595}$  of 0.313 and stored  
19 at 4° C for 1 hour before utilization in various “live cell” mixtures. The other half was  
20 suspended in 4% formaldehyde (freshly prepared from paraformaldehyde), shaken at 140  
21 rpm for 30 minutes at 30° C, washed four times by repeated centrifugation and  
22 resuspension in MM, and resuspended in MM to an  $\text{OD}_{595}$  of 0.31. Measured (1.8-2.2 ml)  
23 portions of each preparation were also centrifuged at 15,000 x g for 5 min and the pellets  
24 frozen for assays of protein and DNA. At time 0 of the experiment, 8 ml portions of each  
25 cell preparation were mixed with either (a) 8 ml of freshly-prepared 12  $\mu\text{M}$  non-  
26 radioactive  $\text{CrCl}_3$  containing  $^{51}\text{CrCl}_3$  radiotracer (26.49 GBq/mg; 759 MBq/ml) or (b) 8 ml  
27 of 12  $\mu\text{M}$  non-radioactive  $\text{Na}_2\text{CrO}_4$  and  $\text{Na}_2^{51}\text{CrO}_4$  radiotracer (17.34 GBq/mg; 29.1  
28 MBq/ml), yielding final mixtures with 6  $\mu\text{M}$  Cr and an  $\text{OD}_{595}$  of 0.155 (6.3 mg protein/ml;  
29 214 ng DNA/ml). The mixtures were shaken at 140 rpm and 30° C in 50 ml Falcon tubes  
30 (loosened caps) that were inclined at 45 degrees from the vertical. To determine the total  
31 radioactivity in each mixture, 1 ml was removed and directly added to 19 ml of water and  
32 0.5 ml of concentrated  $\text{HNO}_3$  in duplicate counting vials. To count the radioactivity in  
33 cellular (centrifugal pellet) fractions after 0, 1.5, 3, and 19 hours of equilibration of live or  
34 killed cells with  $\text{CrCl}_3$  or  $\text{Na}_2\text{CrO}_4$ , duplicate 2 ml samples at each time point were  
35 transferred to 2 ml microfuge tubes and centrifuged at 16000 x g for 4 minutes. Each cell  
36 pellet was washed by 4 cycles of sequential centrifugation and resuspension in MM. The  
37 pellet from the fourth centrifugal step was resuspended in 2 ml of MM and transferred to a  
38 counting vial containing 0.5 ml of concentrated  $\text{HNO}_3$ . The volume was then brought to  
39 20 ml with water. The contents of each vial were subjected to wet ashing by the addition  
40 of 20 mg of  $\text{KMnO}_4$  solid per vial, equilibration for 3 days at ambient temperature, and  
41 subsequent addition of a few drops of undiluted hydroxylamine. Cr-51 was measured with  
42 an HpGe gamma detector (Ortec GMX-15185 type n), with 15% efficiency for Co-60  
43 compared to a 3 inch reference NaI detector. Full calibration efficiency was carried out  
44 with a multi-elements source (210Pb, 241Am, 109Cd, 57Co, 139Ce, 51Cr, 133Ba, 88Y  
45 and 60Co). Calculation was performed using the Canberra GENIE2000 program.  
46  
47

1 **2.5. Effect of CrCl<sub>3</sub> concentration on morphology and staining at non-reducing**  
2 **conditions.**

3 Strain MR-1 and its *ccmC* mutant were grown in LB to an OD<sub>600</sub> of 0.3-0.5 to minimize  
4 the initial number of dead cells. The cultures were centrifuged at 2,080 x g during 10 min  
5 at 4°C, resuspended in HSM pH 7.2, and centrifuged as before. The cell pellets were then  
6 resuspended in HSM pH 6 and diluted to OD<sub>600</sub>= 0.1 in 100 ml flasks each containing 50  
7 ml of HSM pH 6, but supplemented with varying concentrations of CrCl<sub>3</sub>. Flasks were  
8 incubated at room temperature (~25° C) without shaking. At timed intervals, each flask  
9 was gently swirled and 1 ml was removed for staining.

10  
11 **2.6. Effect of stimulated growth on CrCl<sub>3</sub> toxicity.** *S. oneidensis* MR-1 was grown to an  
12 OD<sub>600</sub> of 0.5 in LB broth, centrifuged at 2,080 x g for 15 minutes at 4° C, resuspended in  
13 HSM at pH 6, centrifuged as before and resuspended to an OD<sub>600</sub> of 0.1 in HSM, pH 6.  
14 Portions (100 ml) were transferred to 250-ml flasks that were incubated without shaking at  
15 room temperature (~25° C). At hour 0, one flask was supplemented to 200 µM CrCl<sub>3</sub>,  
16 whereas the other was not. At 3 hours, both cultures were amended by the addition of 2  
17 ml of 20-times-concentrated LB broth and further incubated. Samples for plate counting  
18 and staining were taken at 0, 2.5 and 6 hours after the addition of Cr. To concentrate  
19 samples to a higher OD for staining, 1ml samples were centrifuged at 1,560 x g for 15  
20 minutes at room temperature during the staining time; 0.8 ml of each supernate was  
21 removed and the pellet was gently resuspended in the remaining 0.2 ml for microscopic  
22 observation and photography.

23  
24 A replicate experiment with the same protocol as above was performed with the  
25 *ccmC* mutant, for which chromate effects were also tested. At 0 hour, the washed culture  
26 in HSM galactose pH 6 was split into three portions. Each portion was amended to a final  
27 concentration of one of the following: 100 µM CrCl<sub>3</sub>, 100 µM Na<sub>2</sub>CrO<sub>4</sub>, or no Cr.  
28 Sampling, LB addition, and staining were as described above.

29  
30  
31 **3. RESULTS**

32  
33 **3.1. Cr(VI) reduction kinetics for wild type *S. oneidensis* MR-1 and its *ccmC* mutant.**

34 To compare the ability of the *ccmC* mutant to reduce Cr(VI) with that of wild type MR-1,  
35 we examined the kinetics of Cr(VI) removal from solution. This experimental strategy is  
36 based on the fact that Cr(VI) as chromate is soluble, but its reduction product Cr(III)  
37 rapidly precipitates in aqueous media. Figure 1 presents the data from two experiments,  
38 each involving wild type MR-1, its *ccmC* mutant, and formaldehyde-killed cells. All cell  
39 types were carefully adjusted to the same starting protein concentration, to correct for  
40 slight differences in the OD/protein ratio of wild type MR-1 and the *ccmC* mutant. The  
41 only difference between experiments 1 and 2, which were performed in anaerobic SM  
42 lactate medium, pH 7.2, is that the inoculum culture for experiment 1 had been grown in  
43 LB broth, whereas the inoculum for experiment 2 had been grown in SM lactate, pH 7.2,  
44 and thus was better adapted to maintenance medium (MM).

45  
46 **3.1.1 Lack of Cr(VI) reduction by the *ccmC* mutant.** In both experiments (Fig.  
47 1), wild type strain MR-1 rapidly reduced nearly all of the added 100 µM chromate within  
48 the first 2 hours of the experiment, whereas the data for the *ccmC* mutant were not

1 detectably different from those of formaldehyde-killed cells until after 2 hours. The pre-  
2 growth conditions of the inoculum (experiment 1 in comparison with experiment 2)  
3 influenced the rate of Cr(VI) removal from solution by the wild type culture, but did not  
4 change that rate for the *ccmC* mutant or for formaldehyde-inactivated cells.  
5

6 **3.1.2. Kinetic analysis of Cr(VI) reduction.** Several kinetic models were  
7 examined. Neither simple Michaelis-Menton kinetics nor non-competitive inhibition  
8 models fit the data well. The model that best described the data was a dual-enzyme (or  
9 dual-process) model proposed for Cr(VI) reduction by Viamajala et al. (Viamajala et al.,  
10 2003). Table 1 shows the equation for this model and our analysis. The model considers  
11 two distinct mechanisms of Cr(VI) removal from solution: one process that is inhibited by  
12 its product or intermediates (initial rate constant  $r_{do}$  and inactivation constant  $k_d'$  in Table  
13 1) and one is not (initial rate constant  $r_{so}$ , Table 1). Table 1 shows the numerical values  
14 that our modeling has obtained. Values of  $r_{do}$  and  $k_d'$  were large for wild type MR-1, but  
15 low for the *ccmC* mutant. Thus, these parameters describe a reaction or set of reactions  
16 that require *c*-type cytochromes and are the predominant mechanisms of Cr(VI) reduction  
17 in strain MR-1. Interestingly, these reactions appear to be self-inhibitory, as has been  
18 proposed earlier (Viamajala et al., 2003; Bencheikh-Latmani et al., 2007).  
19

20 In contrast, rate " $r_{so}$ " (Table 1) describes a much slower process that predominated  
21 in the mutant (Table 1). This slow removal of Cr(VI) from solution may involve some  
22 combination of active uptake of Cr(VI) into cells and other Cr(VI) removal reactions,  
23 perhaps involving cytoplasmic reductants released by a small percentage of cells that  
24 could have lysed during the experiment. This removal process followed apparent first  
25 order kinetics.  
26

27 In experiment 1, the value of " $r_{do}$ " for wild type was 84 times larger than " $r_{so}$ " for  
28 the *ccmC* mutant. In experiment 2, it was 45 times larger. Values of  $r_{do}$  for wild type and  
29 the mutant differed by a factor of  $10^4$  (Table 1). Thus, Cr(VI) removal from solution was  
30 very much faster for wild type than for the mutant and also was best described by different  
31 mathematical rate terms (Table 1).  
32

33 **3.2. Effects of pH and Cr(VI) reduction on Cr(VI) toxicity.** To test if the self-inhibition  
34 described above for the reduction reaction also applied to viability and if the Cr(III)  
35 reduction product might be involved in this inhibition, we performed plate count assays of  
36 viability in cultures of wild type MR-1 and its *ccmC* mutant after 6 hours of exposure to  
37 100  $\mu$ M Cr(VI) at anaerobic (reducing) growth conditions. In assays of Cr(VI) in solution  
38 at the beginning and the end of the experiment, the wild type strain had reduced 98% of  
39 the added Cr(VI) at pH 7.2 and only 23% at pH 6 during the 6-hour interval. The *ccmC*  
40 mutant removed very little Cr(VI) from solution at either pH, in agreement prior  
41 experiments (Fig. 1) indicating that it did not reduce Cr(VI). These two pH values (6 and  
42 7.2) were tested because our preliminary experiments had shown that inorganic Cr(III)  
43 species were more soluble at pH 6 than at pH 7 (Table 2), in agreement with theoretical  
44 predictions (Baes and Mesmer, 1976). Control experiments confirmed that the utilized  
45 additions of  $\text{CrCl}_3$  or chromate did not affect the culture pH.  
46

47 Figure 2 shows that Cr(VI) was 50 times more lethal to wild type MR-1 than to the  
48 *ccmC* mutant at pH 6, but was equally toxic to both organisms at pH 7.2. Since the salient

1 difference between the two pH conditions is that Cr(III) is more soluble at pH 6 (Table 2),  
2 these data suggest a lethal effect of soluble Cr(III) that is produced by the reduction  
3 reaction.  
4

5 **3.3. Comparison of Cr(III) and Cr(VI) effects at non-reducing conditions.** To  
6 distinguish between the effects of Cr(III) and Cr(VI), we added exogenous Cr(III) as CrCl<sub>3</sub>  
7 and Cr(VI) as chromate to aerobically-grown cultures of the *ccmC* mutant. Viability, total  
8 microscopic counts, and live/dead staining were tested in parallel at various times after the  
9 addition of chromium. Cr concentrations of 30 and 200 μM in the isotonic medium HSM,  
10 pH 6, were tested because 30 μM is near the saturation concentration of inorganic Cr(III)  
11 species in minimal media at pH 6 (Table 2) and 200 μM is a frequent chromate  
12 concentration in Cr(VI) reduction experiments.  
13

14 **3.3.1. Microscopic counts.** Total cells were counted microscopically in samples  
15 that had been stained with the blue-fluorescing dye H33342, which stains both live and  
16 dead cells (McFeters et al., 1995). The number of microscopically detectable cells per ml  
17 did not change substantially (<15%) within the first 6 hours of incubation of any Cr-  
18 supplemented or unsupplemented mixture (Fig. 3A), indicating that there was neither  
19 pronounced growth nor cell lysis, which would have complicated the live/dead cell stains  
20 described in later sections. The maintenance of stable cell numbers was expected for this  
21 experiment, which was conducted in late logarithmic growth phase (OD<sub>600</sub> = 0.5) in the  
22 minimal medium HSM, which allows only very slow replication (1-2 doublings in 24  
23 hours) and also contains isotonic concentrations of NaCl to minimize cell lysis from  
24 osmotic or hydrodynamic stress. By 24 hours, however, some changes were seen; the total  
25 cells in the “no Cr” control culture had increased to 175% of the original value, whereas  
26 that in the various Cr-treated samples had decreased to 50-70% of the original.  
27 Nonetheless, the major conclusion remains that the period between 0 and 6 hours involved  
28 maintenance conditions with nearly constant cell numbers. We will therefore focus on the  
29 first 6 hours in the results below.  
30

31 **3.3.2. Viable counts.** In contrast to microscopic counts (which cannot distinguish between  
32 viable and dead intact cells), viability decreased between 0 and 6 hours in nearly all Cr-  
33 treated samples (Fig. 3B). Figure 3B shows that the rate and magnitude of cell death at pH  
34 6 was strikingly higher for 200 μM CrCl<sub>3</sub> (a 10<sup>4</sup>-fold decrease at 6 hours) than for 200 μM  
35 chromate (a 10-fold decrease at 6 hours). As expected, lower concentrations of CrCl<sub>3</sub> and  
36 chromate had less effect than did 200 μM (Fig. 3B). Replicate experiments (see section  
37 3.6 for an example) consistently showed 10<sup>4</sup>- to 10<sup>6</sup>- fold decreases in the plate count  
38 viability of wild type or *ccmC* mutant cultures exposed to 100 μM CrCl<sub>3</sub>, even for only 3  
39 hours.  
40

41 LB agar plates from chromate-exposed samples exhibited a substantial proportion  
42 of very small colonies (for 200 μM chromate, 20-30% of the colonies were <1/3 normal  
43 size), suggesting that chromate had either: (a) caused mutations in various loci that  
44 affected growth rate, or (b) interacted with proteins in a non-reversible manner leading to  
45 inhibited growth. Surprisingly, all of the colonies from CrCl<sub>3</sub>-treated samples were of a  
46 normal size. This observation may suggest that CrCl<sub>3</sub> had an all-or-none effect so that  
47 either a cell was nonviable or it could grow normally and form a colony of the usual size  
48 and morphology.

### 3.3.3. Carboxy-fluorescein staining of metabolically-active cells.

Carboxyfluorescein diacetate (CFDA), which is not fluorescent in the diacetate form, is actively transported into cells where intracellular esterases cleave the acetate groups from the dye, converting it to a fluorescent form that is trapped inside the cytosol (Joux and Lebaron, 2000; Hoefel et al., 2003). Thus, CFDA stains only those cells with intact cellular membranes, active transport, active esterases and, by inference, at least some enzymatic and other activity (e.g., transmembrane transport).

The CFDA staining pattern of chromate-treated cultures differed markedly from that of  $\text{CrCl}_3$ -treated ones (Fig. 3C). Inhibition by chromate began early and increased with time. In contrast,  $\text{CrCl}_3$  effects on CFDA were greatly delayed. That is, the percentage of CFDA-stained cells after  $\text{Cr(III)}$ -treatment (either 30 or 200  $\mu\text{M}$   $\text{CrCl}_3$ ) at 3 hours was still close to the starting value and within the standard deviation of the control without added Cr (Fig. 3C). At 6 hours, 50% of the cells exposed to 200  $\mu\text{M}$   $\text{CrCl}_3$  still stained with CFDA, although less than 1 cell in  $10^4$  was viable when plated on LB agar (Fig. 3B). Thus, it appears that the loss in viability for  $\text{CrCl}_3$ -treated cells may not be directly linked to a loss of intracellular enzymatic activity. In contrast the loss in viability after chromate treatment (Fig. 3B) was roughly proportional to the decrease in CFDA staining (Fig. 3C), suggesting a role of intracellular effects for chromate, but not for  $\text{CrCl}_3$ .

### 3.3.4 Propidium iodide staining of cells with permeabilized membranes.

Propidium iodide (PI) can only enter cells with damaged cell membranes (i.e., dead cells), where it binds to DNA (Williams et al., 1998).  $\text{CrCl}_3$  had very little effect on PI staining, which was still low for  $\text{CrCl}_3$  even after 22 hours of exposure (Fig. 3D). In contrast, the percentage of PI-stained cells in chromate-treated samples increased with time and was proportional to chromate concentration (Fig. 3D). Thus, chromate seemed to cause more cell membrane damage than  $\text{CrCl}_3$  did during the slow- or no-growth conditions of HSM medium.

**3.3.5 Morphology of stained cells.** Striking changes in morphology were seen with cells exposed to 200  $\mu\text{M}$   $\text{CrCl}_3$  (Fig. 4B), whereas the morphology of chromate-treated cultures (Fig. 4C) resembled that of untreated ones (Fig. 4A).  $\text{CrCl}_3$  exposure appeared to stimulate the appearance of cells with irregularly-positioned, incomplete, or abnormally large cell division septa, as seen in either H33342- (not shown) or CFDA-stained preparations (Fig. 4B). Very few of these modified cells were stained with PI (Fig. 3D). The lack of PI staining, taken together with positive CFDA staining, indicated that most  $\text{CrCl}_3$ -treated cells still had intact cell membranes and were still enzymatically active at 6 hr, although they were morphologically abnormal.

**3.4. Cellular uptake of radio-labeled  $\text{CrCl}_3$  or chromate.** To explore whether the differing toxic effects of  $\text{CrCl}_3$  and chromate might reflect the relative extents of their transport into cells, we tested the uptake of radio-labeled  $\text{CrCl}_3$  and chromate by wild type MR-1 in MM medium, pH 6. To control for Cr adsorption to cell surfaces or for other processes that do not require active metabolism, formaldehyde-inactivated cells were tested at the same  $\text{OD}_{600}$  as the live cells. The data clearly indicated active uptake of  $^{51}\text{Cr}$ -labeled chromate into the cellular fraction, since both the initial uptake rate and final yield of cell-associated chromate was much higher for live than formaldehyde-treated cells (Fig. 5). In contrast, no detectable difference was observed between the accumulation of  $\text{Cr(III)}$

1 by live or formaldehyde-killed cells (Fig. 5). Thus, if active transport of Cr(III) into *S.*  
2 *oneidensis* MR-1 occurs, it cannot be detected above the effect of adsorption or chemical  
3 reaction that does not require active metabolism. Cellular material (live or dead cells)  
4 was required, however, because the accumulation of <sup>51</sup>Cr(III) in the centrifugal pellet  
5 fraction was considerably less in samples without added cells. Also, <sup>51</sup>Cr(III) uptake was  
6 proportional to the concentration of cellular protein. For example, 19-hour samples of  
7 cellular fractions with protein concentrations of 0, 1.66 and 6.34 μg/ml (corresponding to  
8 0, 144, and 472 ng DNA/ml) contained the following percentages of the added CrCl<sub>3</sub>:  
9 1.2%, 6.7%, and 20.5%, respectively. Furthermore, 6 μM added Cr was utilized because  
10 preliminary experiments had indicated the occurrence of very little precipitation of 6 μM  
11 Cr(III) at pH 6 in MM medium (Table 2). Thus, the equal accumulation of Cr(III) in the  
12 cellular fraction of living or dead cultures (Fig. 5) is more likely related to reactions of  
13 Cr(III) with cellular material, perhaps including cell-nucleated precipitation or adsorption,  
14 than to inorganic precipitation.

15  
16 **3.5. Effect of CrCl<sub>3</sub> concentration on morphology and staining at non-reducing**  
17 **conditions.** To further explore the morphological effects reported above, both wild type  
18 MR-1 and its *ccmC* mutant were exposed to 0, 6, 30, 60, 100, and 200 μM CrCl<sub>3</sub> in HSM  
19 medium, pH 6. For ≥30 μM CrCl<sub>3</sub>, both wild type and the mutant showed changes in  
20 cellular morphology after only 1.5 hours of CrCl<sub>3</sub> treatment (Fig. 6, panels C-F). Because  
21 the two strains reacted identically, only the results for the wild type are given in Figure 6.  
22 Each individual green unit in Figure 6 can be interpreted as a discrete membrane-enclosed  
23 entity because CFDA accumulates in the cytosol and cannot readily exit through the cell  
24 membrane (Joux and Lebaron, 2000; Hoefel et al., 2003). The most striking change with  
25 increasing CrCl<sub>3</sub> concentration was the formation of multi-cell chains that contained more  
26 fluorescent units than the occasional two-cell chains in cultures without CrCl<sub>3</sub> (Fig. 6A).  
27 These chains often consisted of fluorescent units of considerably different lengths that  
28 were separated by black bands of more variable size than in cultures without Cr. We  
29 hypothesize that each black band indicates the location of a cell division septum or cell  
30 division attempt and that many cell chains contain irregularly placed septa.

31  
32 **3.6. Effect of stimulated cellular elongation on CrCl<sub>3</sub> toxicity.** At first glance, the data  
33 above present an interesting paradox: CrCl<sub>3</sub>-exposed cells with altered morphologies  
34 exhibited continued CFDA staining when maintained in HSM medium (Fig. 3C, 4, and 6)  
35 but showed a 10<sup>4</sup>-fold loss in viability when plated on LB agar (Fig. 3B). The easiest  
36 explanation of this inconsistency is that cells with abnormal morphology were not able to  
37 divide normally and to form colonies when shifted to LB agar. To test this hypothesis, we  
38 incubated portions of a wild-type MR-1 culture (OD<sub>600</sub> = 0.1) with either 0 or 200 μM  
39 CrCl<sub>3</sub> for 3 hours, which was enough time to achieve abnormal morphology. At 3 hours,  
40 each culture was supplemented by a 1/50 dilution from a 20-fold concentrated stock of LB  
41 broth and incubated for three more hours. Plate counting (Table 3) and viable staining  
42 (Fig. 7) were performed at appropriate times.

43  
44 Table 3 indicates that samples without added Cr did indeed exhibit greater growth  
45 in the second half of the experiment (note the 19-fold increase in cfu/ml during the last 3.5  
46 hours) than in the first half (the 2-fold increase during the first 2.5 hours). Also  
47 anticipated was the large effect of 200 μM CrCl<sub>3</sub> on plate count viability during the first  
48 2.5 hours of the experiment (Table 3), consistent with earlier experiments (Fig. 3B). It is

1 unlikely that Cr carryover into the LB plates can explain the low viability at 2.5 and 6  
2 hours with 200  $\mu\text{M}$   $\text{CrCl}_3$  because the maximum Cr carryover concentrations in the  
3 various utilized plates would have been between  $8 \times 10^{-9}$  M and  $8 \times 10^{-12}$  M (Footnote b,  
4 Table 3), well below the toxic range of  $\text{CrCl}_3$  to strain MR-1 (Bencheikh-Latmani et al.,  
5 2007).

6  
7 Figure 7 presents the percentages of cells stained by CFDA (enzymatically active),  
8 PI (damaged cell membranes) or H33342 but neither PI nor CFDA (intact cell membranes  
9 but enzymatically inactive) before and after LB addition. At 2.5 hours of incubation and  
10 just before the addition of LB, the cultures with and without  $\text{CrCl}_3$  both had >70% of  
11 CFDA-stained cells, <10% of PI-stained cells, and 15-30% of H33342-only stained cells.  
12 However, at 6 hours (3 hours after the addition of LB) CFDA staining had disappeared  
13 almost completely from the  $\text{CrCl}_3$ -containing cultures and had been replaced quantitatively  
14 by PI staining (Fig. 7), whereas the control without Cr still showed the same CFDA  
15 staining as before (Fig. 7). The percentages of H33342-stained cells did not change  
16 markedly between 2.5 and 6 hours in the Cr-treated culture, suggesting that the formation  
17 of PI-stained cells with damaged membranes occurred almost entirely at the expense of  
18 metabolically active, CFDA-staining cells and that most of the enzymatically inactive  
19 H33342-staining cells did not become ruptured. Thus, active enzymes and cellular growth  
20 were needed to cause cell membrane rupture and death after  $\text{CrCl}_3$  treatment.

#### 21 22 4. DISCUSSION

23 In contrast to the widespread conception that Cr(III) is generally not toxic at  
24 biologically-relevant conditions (Cary, 1982; Alcedo and Wetterhahn, 1990; EPA, 1998),  
25 freshly-dissolved  $\text{CrCl}_3$  is shown here to be strongly toxic at pH 6 to *Shewanella*  
26 *oneidensis* MR-1, a Cr(VI)-reducing bacterium that is frequently studied as a model for  
27 microbial remediation of Cr(VI) pollution. This observation supports previous reports that  
28 Cr(III) produced during Cr(VI) reduction by this strain inhibits both its viability and its  
29 continued reduction of Cr(VI) and that ligands that reduce the bioavailability of the soluble  
30 inorganic ions of Cr(III) protect against this inhibition (Bencheikh-Latmani et al., 2007).  
31 That the Cr(III) product of the Cr(VI) reduction process is indeed toxic to strain MR-1 is  
32 confirmed by the data in Figure 2 that compare viability after Cr(VI) exposure of wild type  
33 MR-1 and its *ccmC* mutant, which cannot reduce Cr(VI); Cr(VI) was more toxic to the  
34 wild type than to the mutant at pH 6, where various inorganic ions of Cr(III) such as  
35  $\text{Cr}(\text{OH})_2^{2-}$  and  $\text{Cr}(\text{OH})_2^{1-}$  are soluble, but not at pH 7.2, where Cr(III) ions are less soluble  
36 (Table 2) (Rai et al., 2004; Remoundaki et al., 2007). Furthermore, the kinetics of Cr(VI)  
37 reduction (Fig. 1) by this organism are best modeled by equations (Table 1) in which  
38 cytochrome *c*-dependent Cr(VI) reduction is inhibited by its product, supporting the earlier  
39 kinetic analysis of Viamajala et al. (Viamajala et al., 2003). None of these data exclude  
40 the possibility that various reduction intermediates or other parts of the Cr(VI)-reduction  
41 process might also be inhibitory, as has been proposed for other systems (Daulton et al.,  
42 2007). However, the rapid and spectacular Cr(III) effects reported here are sufficient to  
43 explain most, if not all, of the toxicity observed at our conditions. Because our  
44 experiments involved short exposure times, when cell lysis was minimal (Fig. 3A), they  
45 are more likely to emphasize biological processes than are longer timescale studies, for  
46 which interpretation is complicated by the release of intracellular reductants and additional  
47 chemical reactions.

1           It is possible to define a toxicity pattern that is characteristic of exposure to CrCl<sub>3</sub>  
2 and not to chromate. This Cr(III) toxicity signature, which occurred when freshly-  
3 dissolved CrCl<sub>3</sub> was added to either the wild type strain MR-1 or its reduction-deficient  
4 mutant, involved the appearance of irregularly-spaced membrane-enclosed units of  
5 varying sizes and numbers within single cells or chains of cells (Fig. 4B). This unusual  
6 morphology was not seen in parallel cultures of the *ccmC* mutant exposed to Cr(VI) under  
7 non-reducing conditions (Fig. 4C). The abnormalities appeared as early as 1.5 hours of  
8 CrCl<sub>3</sub> treatment (Fig. 6), depended on CrCl<sub>3</sub> concentration (Fig. 6), and were accompanied  
9 by a viability loss of several orders of magnitude in plate-count assays (Fig. 3B). In  
10 contrast, cultures supplemented with comparable chromate concentrations lost viability  
11 much more gradually and to a lesser degree, based on both plate counting (Fig. 3B) and  
12 vital staining (Fig. 3C and 3D). Although prolonged exposure to Cr(VI) is known to cause  
13 the appearance of aseptate and greatly elongated cells (Chourey et al., 2006), we did not  
14 observe this phenomenon during the short time scale of our experiments, which involved a  
15 minimal medium and insufficient growth rates to demonstrate cellular elongation.  
16 Furthermore, the cellular morphologies of completely aseptate but elongated cells reported  
17 for Cr(VI) exposure (Chourey et al., 2006; Thompson et al., 2007) are quite distinct from  
18 what we observed here for CrCl<sub>3</sub> exposure.

19  
20           Although future studies will be needed to clarify the mechanism of CrCl<sub>3</sub> toxicity  
21 in *S. oneidensis* MR-1, the current data suggest topics to investigate. The predominant  
22 effect did not seem to be intracellular toxicity involving decreased enzymatic function  
23 because CrCl<sub>3</sub>-treated cells continued to fluoresce as brightly with carboxyfluorescein  
24 diacetate (CFDA) (Joux and Lebaron, 2000; Hoefel et al., 2003) as did control untreated  
25 cells, for several hours in a minimal isotonic medium (Fig. 3C, 4, 6). In contrast, parallel  
26 cultures of chromate-exposed cells showed decreased CFDA staining (Fig. 3C), consistent  
27 with the known cellular uptake and intracellular damage resulting from chromate exposure  
28 (Chourey et al., 2006). Initial cell lysis can also be excluded as a mechanism of CrCl<sub>3</sub>  
29 toxicity because most CrCl<sub>3</sub>-treated cells retained CFDA dye and also did not stain with  
30 propidium iodide (Fig. 3D), which can only enter cells with damaged membranes  
31 (Williams et al., 1998). Instead, there appeared to be an abnormal morphology yielding  
32 irregularly spaced, membrane-enclosed units of differing lengths (Fig. 6) that were still  
33 joined together in chains. Some delay or inhibition in the separation of daughter cells may  
34 be the origin of this morphology. An inability to divide normally is also suggested by data  
35 in Figure 7, for which CrCl<sub>3</sub>-treated cells that were metabolically active (but not  
36 elongating) in minimal isotonic medium were stimulated to divide more rapidly by the  
37 addition of LB broth, which stimulated growth. Only 3 hours after the addition of LB,  
38 essentially all of the CrCl<sub>3</sub>-treated cells that had previously stained with CFDA no longer  
39 stained with that dye but instead stained with PI, which can only enter cells with damaged  
40 membranes. This massive cell lysis paralleled the almost complete loss of viability  
41 observed with CrCl<sub>3</sub>-treated cultures were plated on LB agar (Fig. 3). Thus, CrCl<sub>3</sub>-  
42 exposed cells with abnormal morphologies stayed intact as long as they were held in  
43 isotonic minimal media, but rapidly lysed if they were forced to divide, even if the  
44 medium remained isotonic, as it did for Fig. 7. The mechanism by which Cr(III) leads to  
45 this unusual morphology is not readily deducible from this work. However, it is clear that  
46 this morphology ultimately causes cell death.

47

1           Marked pathological changes occurred within the first 1.5 hours of CrCl<sub>3</sub> exposure  
2 and affected all cells equally within a culture (Fig. 4). This situation suggests chemical  
3 reactions at the cell surface rather than more subtle ones requiring prior cellular uptake, an  
4 interpretation that is consistent with the known reactivity of Cr(III) ions, which are likely  
5 to combine and remain associated with the first cellular structure that they encounter.  
6 Indeed, extensive and stable biosorption of Cr(III) by cell walls, lipopolysaccharides and  
7 other surface components has been well documented (Snyder et al., 1978; McLean and  
8 Beveridge, 1990; Volesky and Holan, 1995; Kratochvil and Volesky, 1998). The idea that  
9 cell surface reactions predominate here was supported by our inability to demonstrate  
10 active uptake of <sup>51</sup>Cr(III) in our system, although rapid active uptake of <sup>51</sup>Cr(VI) was  
11 shown, as was the sorption of <sup>51</sup>Cr(III) to formaldehyde-killed cells (Fig. 5).

12  
13           Understanding the mechanisms and manifestations of Cr(III) toxicity is of interest  
14 for many reasons. For example, it determines whether one can find a way to bypass the  
15 self-inhibitory aspects of Cr(VI) reduction to obtain effective microbial bioremediation of  
16 Cr(VI) pollution. Second, it pertains to whether Cr(III) toxicity is a widespread  
17 phenomenon that might affect many organisms in low pH environments or whether it is  
18 limited to bacteria only. Here we describe a morphological signature of Cr(III) toxicity in  
19 *S. oneidensis* MR-1, which can aid the future investigation of these questions.

20  
21  
22

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8

**Table 1.** Modeling of Cr(VI) reduction by *Shewanella oneidensis* MR-1 wild type and *ccmC* mutant with the equation of Viamajala et al. (Viamajala et al., 2003)\*:

$$[Cr(VI)] = [Cr(VI)]_0 - [r_{so} \times t + \frac{r_{do}}{k_d}(1 - \exp(-k_d \times t))]$$

	<b>r<sub>so</sub></b> ( <b>μM Cr(VI)/hr</b> )	<b>r<sub>do</sub></b> ( <b>μM Cr(VI)/hr</b> )	<b>k<sub>d</sub>'</b> ( <b>hr<sup>-1</sup></b> )	<b>R<sup>2</sup></b>
<b>Wild type (Ex2)</b>	0.15**	<b>109</b>	1.32	0.98
<b>Wild type (Ex1)</b>	0.11**	<b>186</b>	2.25	0.98
<b>ccmC mutant (Ex2)</b>	<b>2.4**</b>	<0.01	<0.01	0.97
<b>ccmC mutant (Ex1)</b>	<b>2.2**</b>	<0.01	<0.01	0.92

\*Before analysis, the data were corrected by subtraction of the amounts of Cr(VI) removed from solution by formaldehyde-treated cells (Fig. 1). The model was fitted to the data with Matlab and a trust-region algorithm. For the results of the *ccmC* mutant, a linear fit was also done because the data, after subtraction of the formaldehyde-treated control, showed a single rate.

\*\*Values of **r<sub>so</sub>** for wild type and mutant differ when expressed in units of Cr concentration removed per hour, as in the original model. However, further normalization of **r<sub>so</sub>** to the concentration of Cr(VI) remaining in solution yields values of **r<sub>so</sub>/μM Cr** for wild type and mutant that are  $0.02 \pm 0.005 \text{ hr}^{-1}$  in all cases.

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**Table 2.** Soluble total Cr in filtered samples of MM medium, pH 6 or pH 7, supplemented with CrCl<sub>3</sub> and either uninoculated or inoculated with *Shewanella oneidensis* MR-1. <sup>a</sup>

Added CrCl <sub>3</sub> (μM)	MM <sup>b</sup> pH 6		MM <sup>b</sup> pH 7.2	MR-1 culture <sup>c</sup> In MM, pH 6		Soluble Cr/ Added Cr	
	Soluble Cr <sub>t</sub> <sup>d</sup> (μM)	Soluble Cr(VI) <sup>e</sup> (μM)	Soluble Cr <sub>t</sub> <sup>d</sup> (μM)	Soluble Cr <sub>t</sub> <sup>d</sup> (μM)	Soluble Cr(VI) <sup>e</sup> (μM)	Sterile MM, pH6 <sup>b</sup> (soluble Cr / added Cr)	MR-1 in MM, pH6 <sup>c</sup> (soluble Cr/ added Cr)
<b>0</b>	< 0.02 <sup>f</sup>	< 0.3 <sup>f</sup>	< 0.02 <sup>f</sup>	< 0.02 <sup>f</sup>	< 0.3 <sup>f</sup>	NA <sup>g</sup>	NA <sup>g</sup>
<b>6</b>	<b>4.9 ± 0.3</b>	< 0.3	< 0.02	<b>3.7 ± 1.1</b>	< 0.3	0.82	0.61
<b>60</b>	<b>15.9 ± 0.5</b>	< 0.3	< 0.02	<b>25.3 ± 2</b>	< 0.3	0.27	0.42
<b>300</b>	<b>26.9 ± 0.4</b>	< 0.3	NT <sup>g</sup>	<b>114 ± 2</b>	< 0.3	0.09	0.38
<b>3000</b>	<b>33.4 ± 0.9</b>	< 0.3	NT	<b>648 ± 6</b>	< 0.3	0.01	0.21

6  
7 <sup>a</sup> After equilibration for 24 hours at 20° C, with shaking at 140 rpm.  
8 <sup>b</sup> Sterile (uninoculated) MM medium.  
9 <sup>c</sup> Initial cell density of 5 x 10<sup>6</sup> cells/ml.  
10 <sup>d</sup> Assayed by ICP-OES. Mean and range of duplicate samples (2 of the 4 samples taken; 2  
11 used for ICP, 2 for DPC).  
12 <sup>e</sup> Assayed by the diphenylcarbazide (DPC) method (Urone, 1955). Assays of all duplicate  
13 samples were below the detection limit (0.3 μM) of the assay.  
14 <sup>f</sup> Less than the assay detection limit. <sup>g</sup> NA, not applicable; NT, not tested

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1 **Table 3.** Plate counts of *Shewanella oneidensis* MR-1 cultures grown in the absence or  
 2 presence of 200  $\mu\text{M}$   $\text{CrCl}_3$  in HSM medium and subsequently supplemented with LB broth  
 3 at hour 3 of the experiment.  
 4  
 5

CrCl <sub>3</sub> concentration ( $\mu\text{M}$ )	Colony forming units (cfu) per milliliter (Mean $\pm$ standard deviation <sup>a</sup> )		
	Hour 0 (just before CrCl <sub>3</sub> addition)	Hour 2.5 (before LB broth addition)	Hour 6 (3 hours after LB broth addition)
0	$1.3 \pm 0.1 \times 10^7$	$3.0 \pm 0.2 \times 10^7$	$5.8 \pm 0.3 \times 10^8$
200	$1.3 \pm 0.1 \times 10^7$	$< 10^3$ <sup>b</sup>	$1.8 \pm 0.4 \times 10^3$

6  
 7 <sup>a</sup> From the counting of at least three LB agar plates with 30-300 colonies each.  
 8

9 <sup>b</sup> At this timepoint, all LB plates that had been inoculated with 0.1 ml of various 10-fold  
 10 dilutions between  $10^2$  to  $10^5$  failed to show any detectable colonies, even during prolonged  
 11 incubation. Based on the dilutions used and the volumes plated, the maximum molar  
 12 concentration of carried-over  $\text{CrCl}_3$  in each set of plates would have been between  
 13  $8 \times 10^{-9}$  M ( $10^2$ -fold tube dilution, 0.1ml spread on each 25 ml plate) and  $8 \times 10^{-12}$  M ( $10^5$ -  
 14 fold tube dilution, 0.1ml spread on each 25 ml plate).  
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## FIGURE LEGENDS

**Figure 1.** Chromate reduction, measured as Cr(VI) removal from solution, by wild type *Shewanella oneidensis* MR-1, its *ccmC* mutant, and formaldehyde-killed cells of each strain. Experiments (Ex) 1 and 2, which were performed in parallel in SM medium, differed only in that the original inoculum of Ex 2 had been grown in LB broth, whereas that for Ex 1 had been grown in SM. Diamonds, wild type, Ex 1; Squares, wild type, Ex 2; Circles, *ccmC* mutant, Ex 1; Triangles, *ccmC* mutant, Ex 2; X, formaldehyde-treated wild type; crossed X, formaldehyde-treated *ccmC* mutant. Bars, which are the standard deviations of triplicate biological measurements, are shown only where they exceed the diameters of the data points.

**Figure 2.** Chromate effects on the viability of wild type MR-1 and its *ccmC* mutant in MM at pH 6 and pH 7.2 (A and B) during growth at reducing conditions. Dark bars, hour 0; white bars, after 6 hours of equilibration with or without 100  $\mu$ M Cr(VI) added as Na<sub>2</sub>CrO<sub>4</sub>. Note the logarithmic scale of the y axis. Bars represent standard deviations of triplicate assays of each sample. A replicate experiment is presented in the supplementary information (Sup. Figure 1).

**Figure 3.** Comparison of microscopic counts (A), viable counts (B), carboxyfluorescein diacetate (CFDA) staining (C) and propidium iodide staining (D) of cultures of *S. oneidensis ccmC* mutant exposed to various concentrations of CrCl<sub>3</sub> or chromate in aerobic HSM medium, pH 6. Diamonds, no Cr; Squares, 30  $\mu$ M chromate; Circles, 200  $\mu$ M chromate; triangles, 30  $\mu$ M CrCl<sub>3</sub>; X, 200  $\mu$ M CrCl<sub>3</sub>. Bars indicate standard deviations (see sections 2.0.2 and 2.0.5). A replicate experiment is presented in the supplementary information (Sup. Figure 2).

**Figure 4.** Appearance of CFDA-stained samples of *S. oneidensis* MR-1 *ccmC* mutant after 6 hours of exposure to 200  $\mu$ M Cr(III) or Cr(VI) at pH 6 in the minimal isotonic medium HSM. Fluorescent CF accumulates inside the cytosol and thus stains metabolically active and intact membrane-enclosed units only. A. No Cr. Note that the two nascent daughter cells within dividing cells are of approximately the same length. B. 200  $\mu$ M CrCl<sub>3</sub>. Note the presence of membrane-enclosed units of variable size and the occasional existence of more than two fluorescing units within a single cell. C. 200  $\mu$ M chromate. Note a morphology similar to that in panel A (no Cr), although the cells appear somewhat less fluorescent, consistent with data in Figure 3C. Scale bar corresponds to 10  $\mu$ m.

**Figure 5.** Effect of prior formaldehyde inactivation on the cellular uptake of 6  $\mu$ M <sup>51</sup>Cr-labeled CrCl<sub>3</sub> or chromate by wild type *S. oneidensis* MR-1 in minimal medium, pH 6, under non-reducing conditions. Triangles, formaldehyde-killed cells with chromate. Circles, live cells (not formaldehyde treated) with chromate; Diamonds, formaldehyde-killed cells with CrCl<sub>3</sub>. Squares, live cells with CrCl<sub>3</sub>. Bars indicate ranges of duplicate samples. A replicate experiment is presented in the supplementary information (Sup. Figure 3).

1 **Figure 6.** Appearance of CFDA-stained samples of wild type *S. oneidensis* MR-1 after  
2 1.5 hours of exposure to the indicated concentrations of CrCl<sub>3</sub> in HSM medium, pH 6.  
3 Examples of cells containing membrane-enclosed units of variable size or irregular  
4 distribution are circled. CFDA stains of parallel cultures of the *ccmC* mutant (not shown)  
5 exhibited virtually identical morphological changes to those of the wild type, as can also  
6 be seen by comparison to Figure 4. Scale bar corresponds to 10 μm.

7  
8 **Figure 7.** Staining of wild type MR-1 cultures that had been exposed to 0 or 200 μM  
9 CrCl<sub>3</sub> in HSM medium, pH 6, and subsequently supplemented with LB broth after 3 hours  
10 of CrCl<sub>3</sub> exposure. Green: CFDA staining (enzymatic activity and intact cell membranes).  
11 Red: PI staining (damaged cell membranes). Blue: Lack of staining by either CFDA or PI,  
12 but staining with H33342 (low enzymatic activity with intact membranes). Before LB  
13 addition (0 or 2.5 hours after CrCl<sub>3</sub> addition), both the untreated and CrCl<sub>3</sub>-treated cultures  
14 showed 60-75% of CFDA staining and less than 10% of PI staining. Three hours after LB  
15 addition (6 hours after CrCl<sub>3</sub> addition), the percentage of CFDA-stained cells was  
16 essentially unchanged in the untreated samples, but much lower in the CrCl<sub>3</sub>-treated  
17 samples. At the same time, the percentage of PI-staining cells in the CrCl<sub>3</sub>-treated samples  
18 was large and indicated a roughly quantitative conversion of CFDA-staining cells to PI-  
19 staining ones after LB supplementation of CrCl<sub>3</sub>-treated samples. Because of difficulty in  
20 counting sufficient numbers of cells (especially for PI and H33342), the standard  
21 deviations of the counts were as large as 20% of the mean, but nonetheless the differences  
22 between 0 and 200 μM CrCl<sub>3</sub> at 6 hours were clearly apparent.

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