

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 μ 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 μ 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 CrCl₃, which yielded low μ 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 CrCl₃ is also toxic, and that CrCl₃ 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 CrCl₃ on strain MR-1 differ markedly from
19 those of Cr(VI). In particular, CrCl₃ 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.
24

25 26 **2. MATERIALS AND METHODS**

27 28 **2.0. Cultures, media, and general methods.**

29
30 **2.0.1. Cultures and media.** *Shewanella oneidensis* MR-1, isolated by Myers and Nealson
31 (1988) from anoxic sediments of Lake Oneida, NY, USA, was obtained from Oak Ridge
32 National Laboratory, USA. *S. oneidensis* MR-1 *ccmC* mutant, which was originally
33 designated mutant BG148 (Bouhenni et al., 2005), was kindly provided by D. Saffarini
34 and was always grown in media supplemented with ≥ 100 μ g of kanamycin/ml, with
35 periodic restreaking to prevent the accumulation of revertants. Cultures stored at -80° C
36 were streaked on Luria-Bertani (LB) agar and maintained in LB broth or SM medium
37 (Supplementary Table 1) (Bencheikh-Latmani et al., 2007). To avoid complexation of
38 Cr(III), most experiments involving Cr used a maintenance medium (MM), which
39 contained in grams per liter (Supplementary Table 1): NaCl, 0.68; KCl, 0.3; MgCl₂*6H₂O,
40 0.285; Na₂SO₄, 0.3975; NH₄Cl, 0.15; Na₂HPO₄, 0.0125; CaCl₂, 0.0056; D(+) galactose
41 (Fluka), 20 g; and either HEPES (4-(2-hydroxyethyl)-1-Piperazineethanesulfonic acid) or
42 MES (2-(N-morpholino)ethane sulfonic acid) buffer, 40 g. The pH was adjusted to 7.2
43 (HEPES buffer) or 6 (MES buffer) with NaOH. MM maintained the viability of *S.*
44 *oneidensis*, but provided only very slow growth with protein doubling times of 10-15
45 hours at culture OD₆₀₀ readings of ≥ 0.1 . High salts medium (HSM) was isotonic MM
46 supplemented with 8 g of NaCl per liter (Supplementary Table 1). HSM was utilized to
47 prevent osmotic stress in cultures during dilution from LB broth, which contains 10 g of

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 μ L samples of
7 cultures were appropriately diluted in HSM or MM and spread (25 μ L, 100 μ L, and 250
8 μ 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 μ l samples (depending on cell
11 density) were added to 1 ml of a 15 μ 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 μ m pore size, Sterlitech PCTB0225100) or mixed cellulose ester (MCE, 0.45 μ 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 μ l of each
31 sample were added (in duplicate) to 190 μ 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 μ L portions of each sample was mixed (in
35 duplicate) with 720 μ L of MilliQ water and 200 μ 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 μ M bovine serum albumin (BSA)
38 in 800 μ L of 0.0015% Triton X-100 and 200 μ 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 μ 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 OD₆₀₀ 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 OD₆₀₀ = 0.1 for these strains. Protein was thought to be a more reliable measure of
31 enzymatic content than OD₆₀₀ because the *ccmC* mutant has a slightly different
32 protein/OD₆₀₀ 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 Na₂CrO₄ (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 Na_2CrO_4 to a final concentration of 100 μM . 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 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 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 μM CrCl_3 or Na_2CrO_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 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 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 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 μM non-
26 radioactive CrCl_3 containing $^{51}\text{CrCl}_3$ radiotracer (26.49 GBq/mg; 759 MBq/ml) or (b) 8 ml
27 of 12 μM non-radioactive Na_2CrO_4 and $\text{Na}_2^{51}\text{CrO}_4$ radiotracer (17.34 GBq/mg; 29.1
28 MBq/ml), yielding final mixtures with 6 μM Cr and an 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 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 CrCl_3 or Na_2CrO_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 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 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 CrCl₃ concentration on morphology and staining at non-reducing**
2 **conditions.**

3 Strain MR-1 and its *ccmC* mutant were grown in LB to an OD₆₀₀ 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 OD₆₀₀= 0.1 in 100 ml flasks each containing 50
7 ml of HSM pH 6, but supplemented with varying concentrations of CrCl₃. 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 CrCl₃ toxicity.** *S. oneidensis* MR-1 was grown to an
12 OD₆₀₀ 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 OD₆₀₀ 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 CrCl₃,
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 CrCl₃, 100 μM Na₂CrO₄, 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 μ 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 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 CrCl₃
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 (OD₆₀₀ = 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 CrCl₃ (a 10⁴-fold decrease at 6 hours) than for 200 μM
35 chromate (a 10-fold decrease at 6 hours). As expected, lower concentrations of CrCl₃ and
36 chromate had less effect than did 200 μM (Fig. 3B). Replicate experiments (see section
37 3.6 for an example) consistently showed 10⁴- to 10⁶- fold decreases in the plate count
38 viability of wild type or *ccmC* mutant cultures exposed to 100 μM CrCl₃, 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 CrCl₃-treated samples were of a
46 normal size. This observation may suggest that CrCl₃ 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 CrCl_3 -treated ones (Fig. 3C). Inhibition by chromate began early and increased with time. In contrast, CrCl_3 effects on CFDA were greatly delayed. That is, the percentage of CFDA-stained cells after Cr(III) -treatment (either 30 or 200 μM 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 μM 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 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 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). CrCl_3 had very little effect on PI staining, which was still low for 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 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 μM CrCl_3 (Fig. 4B), whereas the morphology of chromate-treated cultures (Fig. 4C) resembled that of untreated ones (Fig. 4A). 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 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 CrCl_3 or chromate. To explore whether the differing toxic effects of CrCl_3 and chromate might reflect the relative extents of their transport into cells, we tested the uptake of radio-labeled 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 OD_{600} as the live cells. The data clearly indicated active uptake of ^{51}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 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 ⁵¹Cr(III) in the centrifugal pellet
5 fraction was considerably less in samples without added cells. Also, ⁵¹Cr(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 CrCl₃:
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 CrCl₃ 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 CrCl₃ in HSM
19 medium, pH 6. For ≥30 μM CrCl₃, both wild type and the mutant showed changes in
20 cellular morphology after only 1.5 hours of CrCl₃ 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 CrCl₃ concentration was the formation of multi-cell chains that contained more
26 fluorescent units than the occasional two-cell chains in cultures without CrCl₃ (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 CrCl₃ toxicity.** At first glance, the data
33 above present an interesting paradox: CrCl₃-exposed cells with altered morphologies
34 exhibited continued CFDA staining when maintained in HSM medium (Fig. 3C, 4, and 6)
35 but showed a 10⁴-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 (OD₆₀₀ = 0.1) with either 0 or 200 μM
39 CrCl₃ 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 CrCl₃ 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 μM CrCl_3 because the maximum Cr carryover concentrations in the
3 various utilized plates would have been between 8×10^{-9} M and 8×10^{-12} M (Footnote b,
4 Table 3), well below the toxic range of 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 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 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 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 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 CrCl₃
2 and not to chromate. This Cr(III) toxicity signature, which occurred when freshly-
3 dissolved CrCl₃ 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 CrCl₃ treatment (Fig. 6), depended on CrCl₃ 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 CrCl₃ exposure.

19
20 Although future studies will be needed to clarify the mechanism of CrCl₃ 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 CrCl₃-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 CrCl₃
29 toxicity because most CrCl₃-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 CrCl₃-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 CrCl₃-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 CrCl₃-treated cultures were plated on LB agar (Fig. 3). Thus, CrCl₃-
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 CrCl₃ 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 ⁵¹Cr(III) in our system, although rapid active uptake of ⁵¹Cr(VI) was
11 shown, as was the sorption of ⁵¹Cr(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.

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7 Architecture, Civil and Environmental Engineering (ENAC) at EPFL.

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)*:

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

	r_{so} (μM Cr(VI)/hr)	r_{do} (μM Cr(VI)/hr)	k_d' (hr⁻¹)	R²
Wild type (Ex2)	0.15**	109	1.32	0.98
Wild type (Ex1)	0.11**	186	2.25	0.98
ccmC mutant (Ex2)	2.4**	<0.01	<0.01	0.97
ccmC mutant (Ex1)	2.2**	<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_{so} 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_{so}** to the concentration of Cr(VI) remaining in solution yields values of **r_{so}/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 CrCl₃ and either uninoculated or inoculated with *Shewanella oneidensis* MR-1. ^a

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

6
7 ^a After equilibration for 24 hours at 20° C, with shaking at 140 rpm.
8 ^b Sterile (uninoculated) MM medium.
9 ^c Initial cell density of 5 x 10⁶ cells/ml.
10 ^d 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 ^e 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 ^f Less than the assay detection limit. ^g 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 μM CrCl_3 in HSM medium and subsequently supplemented with LB broth
 3 at hour 3 of the experiment.
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 5

CrCl ₃ concentration (μM)	Colony forming units (cfu) per milliliter (Mean \pm standard deviation ^a)		
	Hour 0 (just before CrCl ₃ 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	$1.3 \pm 0.1 \times 10^7$	$< 10^3$ ^b	$1.8 \pm 0.4 \times 10^3$

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

9 ^b 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 CrCl_3 in each set of plates would have been between
 13 8×10^{-9} M (10^2 -fold tube dilution, 0.1ml spread on each 25 ml plate) and 8×10^{-12} M (10^5 -
 14 fold tube dilution, 0.1ml spread on each 25 ml plate).
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2 **FIGURE LEGENDS**
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4 **Figure 1.** Chromate reduction, measured as Cr(VI) removal from solution, by wild type
5 *Shewanella oneidensis* MR-1, its *ccmC* mutant, and formaldehyde-killed cells of each
6 strain. Experiments (Ex) 1 and 2, which were performed in parallel in SM medium,
7 differed only in that the original inoculum of Ex 2 had been grown in LB broth, whereas
8 that for Ex 1 had been grown in SM. Diamonds, wild type, Ex 1; Squares, wild type, Ex
9 2; Circles, *ccmC* mutant, Ex 1; Triangles, *ccmC* mutant, Ex 2; X, formaldehyde-treated
10 wild type; crossed X, formaldehyde-treated *ccmC* mutant. Bars, which are the standard
11 deviations of triplicate biological measurements, are shown only where they exceed the
12 diameters of the data points.
13

14 **Figure 2.** Chromate effects on the viability of wild type MR-1 and its *ccmC* mutant in
15 MM at pH 6 and pH 7.2 (A and B) during growth at reducing conditions. Dark bars, hour
16 0; white bars, after 6 hours of equilibration with or without 100 μ M Cr(VI) added as
17 Na_2CrO_4 . Note the logarithmic scale of the y axis. Bars represent standard deviations of
18 triplicate assays of each sample. A replicate experiment is presented in the supplementary
19 information (Sup. Figure 1).
20

21 **Figure 3.** Comparison of microscopic counts (A), viable counts (B), carboxyfluorescein
22 diacetate (CFDA) staining (C) and propidium iodide staining (D) of cultures of *S.*
23 *oneidensis ccmC* mutant exposed to various concentrations of CrCl_3 or chromate in
24 aerobic HSM medium, pH 6. Diamonds, no Cr; Squares, 30 μ M chromate; Circles, 200
25 μ M chromate; triangles, 30 μ M CrCl_3 ; X, 200 μ M CrCl_3 . Bars indicate standard
26 deviations (see sections 2.0.2 and 2.0.5). A replicate experiment is presented in the
27 supplementary information (Sup. Figure 2).
28

29 **Figure 4.** Appearance of CFDA-stained samples of *S. oneidensis* MR-1 *ccmC* mutant
30 after 6 hours of exposure to 200 μ M Cr(III) or Cr(VI) at pH 6 in the minimal isotonic
31 medium HSM. Fluorescent CF accumulates inside the cytosol and thus stains
32 metabolically active and intact membrane-enclosed units only. A. No Cr. Note that the
33 two nascent daughter cells within dividing cells are of approximately the same length. B.
34 200 μ M CrCl_3 . Note the presence of membrane-enclosed units of variable size and the
35 occasional existence of more than two fluorescing units within a single cell. C. 200 μ M
36 chromate. Note a morphology similar to that in panel A (no Cr), although the cells appear
37 somewhat less fluorescent, consistent with data in Figure 3C. Scale bar corresponds to 10
38 μ m.
39

40 **Figure 5.** Effect of prior formaldehyde inactivation on the cellular uptake of 6 μ M ^{51}Cr -
41 labeled CrCl_3 or chromate by wild type *S. oneidensis* MR-1 in minimal medium, pH 6,
42 under non-reducing conditions. Triangles, formaldehyde-killed cells with chromate.
43 Circles, live cells (not formaldehyde treated) with chromate; Diamonds, formaldehyde-
44 killed cells with CrCl_3 . Squares, live cells with CrCl_3 . Bars indicate ranges of duplicate
45 samples. A replicate experiment is presented in the supplementary information (Sup.
46 Figure 3).
47

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 CrCl₃ 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.
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8 **Figure 7.** Staining of wild type MR-1 cultures that had been exposed to 0 or 200 μM
9 CrCl₃ in HSM medium, pH 6, and subsequently supplemented with LB broth after 3 hours
10 of CrCl₃ 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 CrCl₃ addition), both the untreated and CrCl₃-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 CrCl₃ addition), the percentage of CFDA-stained cells was
16 essentially unchanged in the untreated samples, but much lower in the CrCl₃-treated
17 samples. At the same time, the percentage of PI-staining cells in the CrCl₃-treated samples
18 was large and indicated a roughly quantitative conversion of CFDA-staining cells to PI-
19 staining ones after LB supplementation of CrCl₃-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 CrCl₃ at 6 hours were clearly apparent.
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2 **LITERATURE CITED**
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