Extracellular Reduction of Hexavalent Chromium by Cytochromes MtrC and OmcA of *Shewanella oneidensis* MR-1[▽]

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To characterize the roles of cytochromes MtrC and OmcA of *Shewanella oneidensis* MR-1 in Cr(VI) reduction, the effects of deleting the *mtrC* and/or *omcA* gene on Cr(VI) reduction and the cellular locations of reduced Cr(III) precipitates were investigated. Compared to the rate of reduction of Cr(VI) by the wild type (wt), the deletion of *mtrC* decreased the initial rate of Cr(VI) reduction by 43.5%, while the deletion of *omcA* or both *mtrC* and *omcA* lowered the rate by 53.4% and 68.9%, respectively. In wt cells, Cr(III) precipitates were detected by transmission electron microscopy in the extracellular matrix between the cells, in association with the outer membrane, and inside the cytoplasm. No extracellular matrix-associated Cr(III) precipitates, however, were found in the cytochrome mutant cell suspension. In mutant cells without either MtrC or OmcA, most Cr(III) precipitates were found in association with the outer membrane, while in mutant cells lacking both MtrC and OmcA, most Cr(III) precipitates were found inside the cytoplasm. Cr(III) precipitates were also detected by scanning electron microscopy on the surfaces of the wt and mutants without MtrC or OmcA but not on the mutant cells lacking both MtrC and OmcA, demonstrating that the deletion of *mtrC* and *omcA* diminishes the extracellular formation of Cr(III) precipitates. Furthermore, purified MtrC and OmcA reduced Cr(VI) with apparent k_{cat} values of 1.2 ± 0.2 (mean $\pm$ standard deviation) and 10.2 ± 1 s⁻¹ and K_m values of 34.1 ± 4.5 and 41.3 ± 7.9 μ M, respectively. Together, these results consistently demonstrate that MtrC and OmcA are the terminal reductases used by *S. oneidensis* MR-1 for extracellular Cr(VI) reduction where OmcA is a predominant Cr(VI) reductase.

Hexavalent chromium [Cr(VI)] has been widely used in a variety of industrial and military applications worldwide. Consequently, Cr(VI) exists as the oxyanion chromate (CrO₄²⁻) at many industrial sites, mainly from uncontrolled discharges. In fact, 10% of the National Priorities List (Superfund sites) contain Cr(VI), and Cr(VI) is also a major contaminant found at the U.S. Department of Energy's Hanford Site in eastern Washington (7). Widespread Cr(VI) contamination poses major health concerns for the local environment and biota ranging from microorganisms to humans (1, 4, 10, 29). Thus, environments contaminated with Cr(VI) need to be remediated to mitigate the harmful effects of Cr(VI). The water solubility of Cr (i.e., mobility in the environment) and its toxicity to humans are governed by its oxidation state. Although Cr oxidation states range from +6 to -2, its most stable states found in the environment are +6 and +3. In contrast to the highly water-soluble and toxic Cr(VI), Cr(III) is much less soluble in water, where it typically forms (hydr)oxides in the absence of complexing ligands and is much less toxic to humans. Reductive transformation of Cr(VI) to Cr(III), therefore, is an established method to remediate Cr(VI) contamination (31). Because many bacteria can reduce Cr(VI) to Cr(III) aerobically

and/or anaerobically, bacterially mediated Cr(VI) reduction is proposed as one of the strategies for bioremediation of Cr(VI) contamination (1, 2, 5, 31).

The dissimilatory metal-reducing bacterium *Shewanella oneidensis* MR-1 reduces Cr(VI) (2, 11). The reduced Cr(III) forms nanometer-sized particles that can be detected on the bacterial cell surfaces and in the cytoplasm by electron microscopy (EM) (5, 16–18). Global transcriptomic analysis revealed upregulation of 83 genes when *S. oneidensis* MR-1 was treated with 100 μ M Cr(VI) as the sole electron acceptor (2). The upregulated genes included those encoding MtrA, MtrB, MtrC, and OmcA that were involved in the extracellular reduction of solid ferric iron [Fe(III)] (hydr)oxides and metal contaminants uranium [U(VI)] and technetium [Tc(VII)]. Subsequent analysis of transposon and/or gene replacement mutants of *S. oneidensis* MR-1 without functional MtrC, MtrA, and/or MtrB shows that, compared to the results for the wild type (wt), the mutants exhibit attenuated Cr(VI) reduction rates, suggesting the involvement of these proteins in Cr(VI) reduction (2).

Working in concert with the inner membrane tetraheme c-type cytochrome (cyt c) CymA, MtrA, MtrB, MtrC, and OmcA form a network that facilitates electron transfer from the quinone/quinol pool in the inner membrane across the periplasmic space, through the outer membrane, and to the surface of Fe(III) oxides (20, 25, 26). MtrA is a periplasmic decaheme cyt c that forms a tight complex with the trans-outer membrane protein MtrB through which MtrA is believed to

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transfer electrons across the outer membrane directly to MtrC (9). MtrC and OmcA are the outer membrane decaheme cyt *c*'s that are localized on bacterial cell surfaces, where they transfer electrons to Fe(III) oxides either directly via binding, indirectly via electron shuttle flavins, or both (12, 15, 19, 21, 23). Despite recent advances in understanding the roles of MtrC and OmcA in Fe(III) oxide reduction and previous indications that MtrC is involved in Cr(VI) reduction, the exact role of MtrC in the latter is still unclear. Furthermore, whether OmcA is involved in Cr(VI) reduction has never been investigated.

To characterize the roles of MtrC and OmcA in Cr(VI) reduction by *S. oneidensis* MR-1, we measured the effects of deleting *mtrC* and/or *omcA* on Cr(VI) reduction and the cellular locations of reduced Cr(III) precipitates. We found that deletions of these genes had negative effects on *S. oneidensis* MR-1's ability to reduce Cr(VI). We also found that the deletion of both *mtrC* and *omcA* diminished the extracellular formation of Cr(III) precipitates. Furthermore, purified MtrC and OmcA displayed Cr(VI) reductase activity, and OmcA reduced Cr(VI) nearly an order of magnitude faster than MtrC. Together, these results consistently demonstrate that MtrC and OmcA are the extracellular terminal reductases of Cr(VI).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. oneidensis* MR-1 cyt *c* deletion mutants with the mutations $\Delta mtrC$, $\Delta omcA$, and $\Delta mtrC\text{-}\Delta omcA$ have been described in a previous study (19). *S. oneidensis* MR-1 wt and the various mutants used were routinely cultured at 30°C in dextrose-free tryptic soy broth (TSB; Difco, Lawrence, KS). Kanamycin is used at 50 $\mu\text{g}/\text{ml}$.

Cr(VI) reduction. The kinetics of Cr(VI) reduction and the locations of reduced Cr(III) precipitates on and in wt and mutant cells were determined by performing a resting-cell assay. TSB cultures (50 ml) were grown aerobically for 16 h at 30°C at 100 rpm and harvested by centrifugation at $5,000 \times g$ for 5 min. Under these conditions, no growth defect was observed for the mutants used. Cells were washed once in an equal volume of 30 mM sodium bicarbonate buffer (pH 8) at 4°C. Following centrifugation, the cells were resuspended in the bicarbonate buffer at a density of 2×10^9 cells/ml and purged for 10 min with mixed $\text{CO}_2:\text{N}_2$ (80:20) gas. Cr(VI) reduction assays contained 30 mM sodium bicarbonate, pH 8, 0.2 mM K_2CrO_4 (Sigma, St. Louis, MO), and 10 mM sodium lactate that was purged with the mixed $\text{CO}_2:\text{N}_2$ gas and sealed with thick butyl rubber stoppers. Kinetic studies were initiated by adding the purged bacterial cells at a final density of 2×10^8 cells/ml. The same amount of heat-killed wt cells was added as a negative control. The reactions were carried out at 30°C with horizontal incubation at 25 rpm. At predetermined time points, the amount of soluble Cr(VI) remaining in the reaction mixtures was analyzed by the diphenylcarbazide method as previously described (27).

Transmission electron microscopy. After harvesting by centrifugation, bacterial cells were fixed in 2.5% glutaraldehyde. The fixed cells were dehydrated in ethanol series and embedded in LR White resin (Electron Microscopy Sciences, Hatfield, PA). The embedded cells were then sectioned by using an ultramicrotome (Leica, Bannockburn, IL) (13). All sample preparation steps were carried out in an anaerobic chamber. Ultrathin sections were examined at 120 kV using a Tecnai T12 transmission EM (TEM) equipped with LaB6 filament (FEI, Hillsboro, OR). Images were digitally collected and analyzed using DigitalMicrograph software (Gatan, Inc., Pleasanton, CA). Elemental analysis was performed by using an energy-dispersive X-ray spectroscopy (EDX) system (Oxford Instruments, Abingdon, United Kingdom) equipped with a SiLi detector coupled to the JEOL 2010 high-resolution TEM, and spectra were analyzed with ISIS software.

Scanning electron microscopy. About 3 μl of the glutaraldehyde-fixed cells were dropped on a clean coverslip to make a dry film. The film was washed carefully with ultrapure water. Once dry, the film was coated with an electrically conductive thin carbon layer of nanometer thickness for scanning EM (SEM) imaging; images were collected using an FEI Inspect F SEM with a spatial resolution of ~ 1 nm. Secondary electrons were probed to get the SEM image

FIG. 1. Cr(VI) reduction kinetics of *S. oneidensis* MR-1 wild type (wt) and the mutants without MtrC and/or OmcA. The reduction of 200 μM Cr(VI) was determined for the wt and mutants without MtrC ($\Delta mtrC$), OmcA ($\Delta omcA$), or MtrC and OmcA ($\Delta mtrC\text{-}\Delta omcA$) using 10 mM sodium lactate as the electron donor. Heat-killed cells were used as a negative control. The values reported are the means and standard deviations of triplicate measurements.

under a typical acceleration voltage of 20 kV. An EDX system (INCA PentaFET $\times 3$, Oxford Instruments, Abingdon, United Kingdom) was also attached to the microscope.

Protein film voltammetry. To determine whether they reduced Cr(VI), recombinant MtrC and OmcA were purified by following procedures described previously (22, 24, 32). Briefly, recombinant MtrC and OmcA were overexpressed in *S. oneidensis* MR-1 cells. Following cell lysis and ultracentrifugation, membrane-associated MtrC and OmcA were solubilized with detergent. The solubilized MtrC and OmcA were then purified by immobilized metal ion affinity chromatography.

The Cr(VI) reductase activity of purified cyt *c*'s was measured by using protein film voltammetry (PFV). Protein films were prepared using a Hamilton syringe to apply several microliters of 40 μM MtrC or OmcA in 50 mM HEPES, 100 mM NaCl, and 0.5% (wt/vol) 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS), pH 7, to the electrode surfaces. The pH effects on the voltammograms were measured in a buffer of 50 mM 2-morpholineethanesulfonic acid (MES) (pH 6), HEPES (pH 8), *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) (pH 9), or 3-(cyclohexyl)-1-aminopropanesulfonic acid (CAPS) (pH 10 and pH 11) with 100 mM NaCl. The voltammetric responses of cyt *c*'s to different concentrations of K_2CrO_4 were measured in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4 , 2 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, pH 7.4) with a scan rate of 30 mV s^{-1} and an electrode rotation rate of 3,000 rpm. The steady-state reduction rate constants were then calculated (8, 30).

RESULTS

In vivo Cr(VI) reduction. To characterize the roles of MtrC and OmcA in Cr(VI) reduction, we first comparatively measured the whole-cell rates of Cr(VI) reduction by the wt and three different gene deletion mutants with the mutations $\Delta mtrC$, $\Delta omcA$, and $\Delta mtrC\text{-}\Delta omcA$. Under the test conditions, all strains reduced Cr(VI). At 4 h, the reduction rate was $19.3 \pm 0.4 \mu\text{M Cr(VI) h}^{-1}$ (mean $\pm$ standard deviation; $n = 3$) for the wt. At the same time, the reduction rate was $10.9 \pm 1 \mu\text{M Cr(VI) h}^{-1}$ ($n = 3$) for the $\Delta mtrC$ strain (i.e., 56.5% of

FIG. 2. Cellular locations of Cr(III) precipitates in *S. oneidensis* MR-1 wild type (wt) and the mutants without MtrC and/or OmcA. (A to D) TEM images of wt (A and B) and mutants without OmcA (C) or MtrC and OmcA (D) at 120 h. (E and F) SEM images of the wt (E) and the mutant without MtrC and OmcA (F) at 120 h. Arrows indicate the Cr(III) precipitates. The images shown are representative of 10 different images.

that for the wt), whereas the reduction rates were 9.0 ± 0.3 and $6.0 \pm 1.8 \mu\text{M Cr(VI)} \text{ h}^{-1}$ ($n = 3$) for the ΔomcA and $\Delta\text{mtrC-}\Delta\text{omcA}$ mutants, respectively; these rates were 46.6% and 31.1% of the reduction rate for the wt. Additional Cr(VI) was reduced by all tested strains between 4 and 24 h (Fig. 1). After that, only a small amount of Cr(VI) was further reduced between 24 and 120 h (data not shown). No Cr(VI) reduction was observed in the heat-killed-cell control (Fig. 1). Together, these results demonstrate that deletion of *mtrC* and/or *omcA* negatively affects *S. oneidensis* MR-1's ability to reduce Cr(VI).

Cellular locations of reduced Cr(III) precipitates. We then used TEM to examine the cellular locations of reduced Cr(III)

precipitates in wt and mutant cells. In wt cells, electron-dense precipitates were detected by TEM in the extracellular matrix between the cells and in association with the outer membrane at 24 h following reduction. At 48 h, scattered precipitates were also present inside the cytoplasm (data not shown). More precipitates were detected in these locations at 120 h (Fig. 2A and B). SEM analysis also showed that wt cells collected at 120 h were coated with precipitates (Fig. 2E). Analyses of these cell envelope-associated precipitates with TEM- and SEM-coupled EDX confirmed that they were rich in Cr (Fig. 3A), suggesting that similar precipitates observed in the extracellular matrix between the cells and inside cells were also Cr precipitates. In

FIG. 3. EDX-based elemental analysis of the electron-dense materials detected by TEM. (A and B) TEM images of the mutant cells without MtrC (A) or MtrC and OmcA (B). Circles highlight the areas analyzed by EDX. Insets: EDX spectra indicate the presence of Cr in the electron-dense particulates associated with the outer membrane (A) but not in the clustered materials inside bacterial cells (B).

contrast, another form of electron-dense materials found inside the cytoplasm of the wt and all mutant cells did not contain detectable Cr by EDX analysis (Fig. 3B). These materials were always clustered together in the cytoplasm, which was distinguishable from the Cr-containing precipitates that were in the extracellular matrix, in association with the outer membrane, and scattered inside the cytoplasm (Fig. 2A to D and 3A). The nature of these electron-dense materials lacking Cr remains unclear. Because the oxidation state of the *S. oneidensis* MR-1-reduced Cr under similar conditions was +3 (5, 16, 18), the Cr-containing precipitates detected by EM were assumed also to be Cr(III).

Different from the results for wt cells, no extracellular matrix-associated Cr(III) precipitates were detected in the mutant resting-cell suspensions during the time course of study. Furthermore, the outer membrane-associated and/or cytoplasmic Cr(III) precipitates were only found in association with the mutant cells after 120 h. In the mutant cells lacking either OmcA or MtrC, most of the Cr(III) precipitates were found in association with the outer membrane. Some precipitates were also found inside the cytoplasm (Fig. 2C and 3A). Cr(III) precipitates were detected on the surfaces of $\Delta mtrC$ or $\Delta omcA$ cells by SEM (data not shown). In $\Delta mtrC$ - $\Delta omcA$ cells, most of the precipitates were detected in the cytoplasm (Fig. 2D). Furthermore, no Cr(III) precipitates were observed by SEM on the surface of $\Delta mtrC$ - $\Delta omcA$ cells (Fig. 2F), demonstrating that the deletion of both *mtrC* and *omcA* diminished the extracellular formation of Cr(III) precipitates.

In vitro Cr(VI) reduction. To determine whether MtrC and OmcA could serve as Cr(VI) reductases, we purified MtrC and OmcA individually and then used PFV to measure their enzymatic activity toward K_2CrO_4 . Under noncatalytic conditions, both MtrC and OmcA films displayed cyclic voltammograms similar to those reported previously (Fig. 4A) (6, 8). Electrode rotation or transfer of the protein-coated electrodes to fresh buffer electrolyte solution did not change the voltammograms, which demonstrates that the voltammograms observed can be attributed to the direct redox transformation of the adsorbed proteins. Likewise, changing the pH of the buffer electrolyte solution to alkaline conditions resulted in a negative shift of

noncatalytic signals of the MtrC film (Fig. 4B) (8). These results all show that the protein films prepared in this study exhibit redox properties that are similar to those reported previously.

Under catalytic conditions, both MtrC and OmcA films also responded electrocatalytically to the presence of K_2CrO_4 in a manner distinct from the responses obtained from bare electrodes. To avoid the interference of nonspecific current from the bare electrodes, reduction currents at -330 mV of MtrC

FIG. 4. Noncatalytic-protein-film voltammetry of MtrC. (A) Cyclic voltammogram of adsorbed MtrC. The red line is the electrode response in the absence of an MtrC protein film. The buffer electrolyte was PBS buffer, pH 7.4, the scan rate was 30 mV s^{-1} , and the temperature was 273 K. (B) The effect of pH on MtrC cyclic voltammetry. Representative baseline-subtracted, normalized response of MtrC in 100 mM NaCl, 50 mM MES, HEPES, CHES, and CAPS at pH 6, 8, 9, 10, and 11 as indicated, with a scan rate of 30 mV s^{-1} and a temperature of 273 K. SHE, standard hydrogen electrode.

FIG. 5. Catalytic-protein-film voltammetry of MtrC and OmcA. The buffer electrolyte was PBS, the scan rate was 30 mV s^{-1} , the electrode rotation was 3,000 rpm, and the temperature was 273 K. (A) Typical cyclic voltammograms from an MtrC film in 0, 0.8, 2, 4, 8, 18, 36, 64, and $100 \mu\text{M K}_2\text{CrO}_4$. The electrochemical potential (-0.33 V) where catalytic current was analyzed is illustrated by a dashed line. Inset: variation of the magnitude of the catalytic current, measured at -0.33 V , with the K_2CrO_4 concentration. The line shows the catalytic current arising from a Michaelis-Menten type of enzymatic kinetics with a K_m value of $34.1 \pm 4.5 \mu\text{M}$ and a k_{cat} value of $1.2 \pm 0.1 \text{ s}^{-1}$. (B) Typical cyclic voltammograms from an OmcA film in 0, 0.8, 2, 4, 8, 18, 36, and $64 \mu\text{M K}_2\text{CrO}_4$. The electrochemical potential (-0.33 V) where catalytic current was analyzed is illustrated by a broken line. Inset: variation of the magnitude of the catalytic current, measured at -0.33 V , with the K_2CrO_4 concentration. The line shows the catalytic current arising from a Michaelis-Menten type of enzymatic kinetics with a K_m value of $41.3 \pm 7.9 \mu\text{M}$ and a k_{cat} value of $10.2 \pm 1.0 \text{ s}^{-1}$. SHE, standard hydrogen electrode.

and OmcA films were used to determine their reduction rates (Fig. 5) (8). As shown in Fig. 5, the reduction currents of MtrC and OmcA films increased with increasing K_2CrO_4 concentration. The saturating behavior of the increased currents was also evident with increasing concentrations of K_2CrO_4 . These data were fitted to the Michaelis-Menten equation, which yielded apparent turnover numbers (k_{cat}) of 1.2 ± 0.1 and $10.2 \pm 1 \text{ s}^{-1}$ and apparent equilibrium constants (K_m) of 34.1 ± 4.5 and $41.3 \pm 7.9 \mu\text{M}$ for MtrC and OmcA, respectively. Based on these results, the apparent second-order rate constants (k_{cat}/K_m) were calculated as $3.5 \times 10^4 \text{ s}^{-1}\text{M}^{-1}$ for MtrC and $2.5 \times 10^5 \text{ s}^{-1}\text{M}^{-1}$ for OmcA (Table 1). All these results clearly show that purified MtrC and OmcA are functional Cr(VI) reductases and that OmcA reduces Cr(VI) nearly 10 times faster than MtrC.

DISCUSSION

To determine the roles of MtrC and OmcA in Cr(VI) reduction by *S. oneidensis* MR-1, we measured the effects of deleting *mtrC* and/or *omcA* on Cr(VI) reduction and the cellular locations of reduced Cr(III) precipitates. We found that, compared to the reduction rate of the wt, deletion of *mtrC* and/or *omcA* lowered the initial rates of Cr(VI) reduction by 43.5 to 68.9% and deletion of both *mtrC* and *omcA* diminished the formation of extracellular Cr(III) precipitates. *In vitro* characterization of purified MtrC and OmcA showed that both

cyt *c*'s reduced Cr(VI) with similar K_m values but different k_{cat} values. Together, these results consistently demonstrate that MtrC and OmcA are the terminal reductases that reduce Cr(VI) extracellularly.

The results of this study are not only consistent with those reported previously but also provide new insight into the roles of these cyt *c*'s in Cr(VI) reduction. Previous investigations showed only that the mRNA levels of *mtrC* and *omcA* increased under the Cr(VI) reducing condition and that replacement of the *mtrC* gene with an antibiotic-resistant gene lowered the Cr(VI) reduction rate (2). Herein, we determine that deletion of *mtrC* and/or *omcA* decreases *S. oneidensis* MR-1's ability to reduce Cr(VI), demonstrating the direct involvement of both MtrC and OmcA in Cr(VI) reduction. The results further reveal that the deletion of *omcA* or both *mtrC* and *omcA* has greater negative effects on *Shewanella*'s ability to reduce Cr(VI) than the deletion of *mtrC* alone.

In previous studies, the locations of reduced Cr(III) precipitates in *S. oneidensis* MR-1 cultures varied depending on the experimental conditions. When $100 \mu\text{M Cr(VI)}$ was used as the sole terminal electron acceptor for 30 days, the Cr(III) was only found outside *S. oneidensis* MR-1 cells (5). When $200 \mu\text{M Cr(VI)}$ was added to cells that had been previously grown with 5 mM nitrate as the sole electron acceptor for 16 h, both extracellular and intracellular Cr-containing nanoparticles were detected in the *S. oneidensis* MR-1 cells at 48 h after the addition of Cr(VI) (16). In this study, reduced Cr(III) precipitates were found both inside and outside the *S. oneidensis* MR-1 cells after reduction of $200 \mu\text{M Cr(VI)}$ for 48 h. Thus, treatment of MR-1 cells with $200 \mu\text{M Cr(VI)}$ significantly enhanced intracellular precipitation of Cr(III). The finding that the elimination of MtrC and OmcA diminishes the Cr(III) precipitates external to the cells emphasizes the role of both MtrC and OmcA in extracellular reduction of Cr(VI).

The roles of MtrC and OmcA in extracellular reduction of

TABLE 1. Michaelis-Menten kinetics constants for reduction of Cr(VI) by MtrC and OmcA

Cytochrome	k_{cat}	$K_m (\mu\text{M})$	$k_{cat}/K_m (\text{s}^{-1}\text{M}^{-1})$
MtrC	1.2 ± 0.1	34.1 ± 4.5	3.5×10^4
OmcA	10.2 ± 1.0	41.3 ± 7.9	2.5×10^5

U(VI) and Tc(VII) have been previously reported. The U(IV) and Tc(IV) precipitates resulting from the reduction of U(VI) and Tc(VII) by MtrC and OmcA were found in association with bacterial outer membranes and/or in extracellular polymeric substances where MtrC and OmcA were localized (13, 14). Previous results also showed that MtrC and OmcA were released to the growth medium by *S. oneidensis* MR-1 cells (23). Thus, it is not surprising that MtrC/OmcA-reduced Cr(III) precipitates were found not only in association with the outer membrane but also in the extracellular matrix between the cells.

The measured k_{cat} values for Cr(VI) reduction by MtrC and OmcA suggest that OmcA reduces Cr(VI) nearly an order of magnitude faster than MtrC. Consistent with this *in vitro* measurement, the $\Delta mtrC$ mutant reduces Cr(VI) faster than the $\Delta omcA$ mutant. The observation that the $\Delta omcA$ and $\Delta mtrC$ - $\Delta omcA$ mutants display similar Cr(VI) reduction kinetics also indicates a dominant role of OmcA in Cr(VI) reduction. The measured K_m values of 34.1 ± 4.5 and 41.3 ± 7.9 μ M for MtrC and OmcA, respectively, are within the range of previously measured half-saturation coefficients (i.e., 29 to 88 μ M) for reducing Cr(VI) by *S. oneidensis* MR-1 cells (16). This measured apparent binding affinity of MtrC and OmcA to Cr(VI) may provide a plausible explanation for the observations that *S. oneidensis* MR-1 reduces Cr(VI) at sub-mM concentrations (2, 5, 16, 18, 28, 29).

Extracellular reduction of Cr(VI) by MtrC and OmcA and the subsequent precipitation of reduced Cr(III) external to *S. oneidensis* MR-1 cells may serve as the integral part of the mechanism for detoxifying Cr under the conditions tested. Similar to previous results (11), most Cr(VI) reduction by all tested strains occurred within 24 h. After that, only a small amount of Cr(VI) was further reduced. The toxic effects of the reduction product, Cr(III), to the bacterial cells were thought to contribute to this observed incomplete reduction of Cr(VI) (16). Subsequent investigations confirmed that while neither Cr(VI), soluble complexed Cr(III), nor Cr(III)(OH)₃ precipitate was toxic to *Shewanella* cells, the uncomplexed Cr(III) lowered the *Shewanella* survival rate. The uncomplexed Cr(III) present in the cytoplasm was believed to exert its toxic effect by interfering with bacterial gene transcription, although the mechanisms of cytoplasmic Cr(III) formation were unknown. It was proposed that the cytoplasmic Cr(III) was produced by either intracellular reduction of Cr(VI) after it entered the cells, the extracellular reduction of Cr(VI) in which the reduced Cr(III) was transported into the cytoplasm after reduction, or both (1). Given that the $\Delta mtrC$ - $\Delta omcA$ mutant still reduces Cr(VI) and that reduced Cr(III) precipitates are found inside cells of the $\Delta mtrC$ - $\Delta omcA$ strain, at least some of the cytoplasmic Cr(III) appears to be generated by intracellular reduction of Cr(VI). A portion of the cytoplasmic Cr(III) may bind to DNA and/or other cellular components to interfere with normal *Shewanella* cellular functions. Extracellular precipitation of Cr(III) following its reduction by MtrC and OmcA can directly decrease the availability of extracellular Cr(VI)/(III), which can indirectly lower the amount of Cr(III) accumulated in the cytoplasm. Consequently, this could help mitigate the toxic effect of Cr(III) on the *Shewanella* cells. Because the K_m values of MtrC and OmcA to Cr(VI) are 34.1 ± 4.5 and 41.7 ± 7.9 μ M, respectively, MtrC-/OmcA-

mediated detoxification should only work optimally at sub-mM concentrations of Cr(VI). They may be ineffective in detoxification when Cr(VI) is at mM concentrations because MtrC and OmcA are oversaturated with respect to their Cr(VI) reductase activity. Probably for this reason, the mRNA and proteins levels of MtrC and OmcA decrease following challenge of *S. oneidensis* MR-1 cells with 1 mM Cr(VI) (3).

In summary, the results obtained from this study collectively support the idea that MtrC and OmcA are the terminal reductases used by *S. oneidensis* MR-1 cells for extracellular reduction of Cr(VI) where OmcA is a predominant Cr(VI) reductase. MtrC-/OmcA-mediated extracellular reduction of Cr(VI) coupled with subsequent extracellular precipitation of Cr(III) can serve as a mechanism for ameliorating the toxic effects of Cr(III) under Cr(VI)-reducing conditions.

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REFERENCES

- Bencheikh-Latmani, R., A. Obratzsova, M. R. Mackey, M. H. Ellisman, and B. M. Tebo. 2007. Toxicity of Cr(III) to *Shewanella* sp. strain MR-4 during Cr(VI) reduction. *Environ. Sci. Technol.* **41**:214–220.
- Bencheikh-Latmani, R., et al. 2005. Global transcriptional profiling of *Shewanella oneidensis* MR-1 during Cr(VI) and U(VI) reduction. *Appl. Environ. Microbiol.* **71**:7453–7460.
- Brown, S. D., et al. 2006. Molecular dynamics of the *Shewanella oneidensis* response to chromate stress. *Mol. Cell. Proteomics* **5**:1054–1071.
- Chourey, K., et al. 2006. Global molecular and morphological effects of 24-hour chromium(VI) exposure on *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.* **72**:6331–6344.
- Daulton, T. L., B. J. Little, K. Lowe, and J. Jones-Meehan. 2002. Electron energy loss spectroscopy techniques for the study of microbial chromium(VI) reduction. *J. Microbiol. Methods* **50**:39–54.
- Firer-Sherwood, M., G. S. Pulcu, and S. J. Elliott. 2008. Electrochemical interrogations of the Mtr cytochromes from *Shewanella*: opening a potential window. *J. Biol. Inorg. Chem.* **13**:849–854.
- Fruchter, J. 2002. In situ treatment of chromium-contaminated groundwater. *Environ. Sci. Technol.* **36**:464A–472A.
- Hartshorne, R. S., et al. 2007. Characterization of *Shewanella oneidensis* MtrC: a cell-surface decaheme cytochrome involved in respiratory electron transport to extracellular electron acceptors. *J. Biol. Inorg. Chem.* **12**:1083–1094.
- Hartshorne, R. S., et al. 2009. Characterization of an electron conduit between bacteria and the extracellular environment. *Proc. Natl. Acad. Sci. U. S. A.* **106**:22169–22174.
- LaVelle, J. M. 1991. Mechanisms of toxicity/carcinogenicity and Superfund decisions. *Environ. Health Perspect.* **92**:127–130.
- Liu, C., Y. A. Gorby, J. M. Zachara, J. K. Fredrickson, and C. F. Brown. 2002. Reduction kinetics of Fe(III), Co(III), U(VI), Cr(VI), and Tc(VII) in cultures of dissimilatory metal-reducing bacteria. *Biotechnol. Bioeng.* **80**:637–649.
- Lower, B. H., et al. 2009. Antibody recognition force microscopy shows that outer membrane cytochromes OmcA and MtrC are expressed on the exterior surface of *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.* **75**:2931–2935.
- Marshall, M. J., et al. 2006. c-Type cytochrome-dependent formation of U(IV) nanoparticles by *Shewanella oneidensis*. *PLoS Biol.* **4**:1324–1333.
- Marshall, M. J., et al. 2008. Hydrogenase- and outer membrane c-type cytochrome-facilitated reduction of technetium(VII) by *Shewanella oneidensis* MR-1. *Environ. Microbiol.* **10**:125–136.
- Meitl, L. A., et al. 2009. Electrochemical interaction of *Shewanella oneidensis* MR-1 and its outer membrane cytochromes OmcA and MtrC with hematite electrodes. *Geochim. Cosmochim. Acta* **2009**:5292–5307.
- Middleton, S. S., et al. 2003. Cometabolism of Cr(VI) by *Shewanella oneidensis* MR-1 produces cell-associated reduced chromium and inhibits growth. *Biotechnol. Bioeng.* **83**:627–637.

17. Myers, C. R., B. P. Carstens, W. E. Antholine, and J. M. Myers. 2000. Chromium(VI) reductase activity is associated with the cytoplasmic membrane of anaerobically grown *Shewanella putrefaciens* MR-1. *J. Appl. Microbiol.* **88**:98–106.
18. Neal, A. L., K. Lowe, T. L. Daulton, J. Jones-Meehan, and B. J. Little. 2002. Oxidation state of chromium associated with cell surfaces of *Shewanella oneidensis* during chromate reduction. *Appl. Surf. Sci.* **202**:150–159.
19. Reardon, C. L., et al. 2010. Role of outer-membrane cytochromes MtrC and OmcA in the biomineralization of ferrihydrite by *Shewanella oneidensis* MR-1. *Geobiology* **8**:56–68.
20. Richardson, D. J. 2000. Bacterial respiration: a flexible process for a changing environment. *Microbiology* **146**(Pt. 3):551–571.
21. Ross, D. E., S. L. Brantley, and M. Tien. 2009. Kinetic characterization of OmcA and MtrC, terminal reductases involved in respiratory electron transfer for dissimilatory iron reduction in *Shewanella oneidensis* MR-1. *Appl. Environ. Microbiol.* **75**:5218–5226.
22. Shi, L., et al. 2006. Isolation of a high-affinity functional protein complex between OmcA and MtrC: two outer membrane decaheme c-type cytochromes of *Shewanella oneidensis* MR-1. *J. Bacteriol.* **188**:4705–4714.
23. Shi, L., et al. 2008. Direct involvement of type II secretion system in extracellular translocation of *Shewanella oneidensis* outer membrane cytochromes MtrC and OmcA. *J. Bacteriol.* **190**:5512–5516.
24. Shi, L., J. T. Lin, L. M. Markillie, T. C. Squier, and B. S. Hooker. 2005. Overexpression of multi-heme C-type cytochromes. *Biotechniques* **38**:297–299.
25. Shi, L., et al. 2009. The roles of outer membrane cytochromes of *Shewanella* and *Geobacter* in extracellular electron transfer. *Environ. Microbiol. Rep.* **1**:220–227.
26. Shi, L., T. C. Squier, J. M. Zachara, and J. K. Fredrickson. 2007. Respiration of metal (hydr)oxides by *Shewanella* and *Geobacter*: a key role for multihaem c-type cytochromes. *Mol. Microbiol.* **65**:12–20.
27. Urone, P. F. 1955. Stability of colorimetric reagent for chromium, S-diphenylcarbazine, in various solvents. *Anal. Chem.* **27**:1354–1355.
28. Viamajala, S., B. M. Peyton, W. A. Apel, and J. N. Petersen. 2002. Chromate reduction in *Shewanella oneidensis* MR-1 is an inducible process associated with anaerobic growth. *Biotechnol. Prog.* **18**:290–295.
29. Viamajala, S., B. M. Peyton, R. K. Sani, W. A. Apel, and J. N. Petersen. 2004. Toxic effects of chromium(VI) on anaerobic and aerobic growth of *Shewanella oneidensis* MR-1. *Biotechnol. Prog.* **20**:87–95.
30. Vincent, K. A., A. Parkin, and F. A. Armstrong. 2007. Investigating and exploiting the electrocatalytic properties of hydrogenases. *Chem. Rev.* **107**:4366–4413.
31. Wang, Y. T. 2000. Microbial reduction of chromate, p. 225–236. In D. R. Lovley (ed.), *Environmental microbe-metal interactions*. ASM Press, Washington, DC.
32. Wigginton, N. S., K. M. Rosso, B. H. Lower, L. Shi, and M. F. Hochella. 2007. Electron tunneling properties of outer-membrane decaheme cytochromes from *Shewanella oneidensis*. *Geochim. Cosmochim. Acta* **71**:543–555.