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Global Transcriptional Profiling of *Shewanella oneidensis* MR-1 during Cr(VI) and U(VI) Reduction†

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Whole-genome DNA microarrays were used to examine the gene expression profile of *Shewanella oneidensis* MR-1 during U(VI) and Cr(VI) reduction. The same control, cells pregrown with nitrate and incubated with no electron acceptor, was used for the two time points considered and for both metals. U(VI)-reducing conditions resulted in the upregulation (≥3-fold) of 121 genes, while 83 genes were upregulated under Cr(VI)-reducing conditions. A large fraction of the genes upregulated [34% for U(VI) and 29% for Cr(VI)] encode hypothetical proteins of unknown function. Genes encoding proteins known to reduce alternative electron acceptors [fumarate, dimethyl sulfoxide, Mn(IV), or soluble Fe(III)] were upregulated under both U(VI)- and Cr(VI)-reducing conditions. The involvement of these upregulated genes in the reduction of U(VI) and Cr(VI) was tested using mutants lacking one or several of the gene products. Mutant testing confirmed the involvement of several genes in the reduction of both metals: *mtrA*, *mtrB*, *mtrC*, and *menC*, all of which are involved in Fe(III) citrate reduction by MR-1. Genes encoding efflux pumps were upregulated under Cr(VI)- but not under U(VI)-reducing conditions. Genes encoding proteins associated with general (e.g., *groL* and *dnaJ*) and membrane (e.g., *pspBC*) stress were also upregulated, particularly under U(VI)-reducing conditions, pointing to membrane damage by the solid-phase reduced U(IV) and Cr(III) and/or the direct effect of the oxidized forms of the metals. This study sheds light on the multifaceted response of MR-1 to U(VI) and Cr(VI) under anaerobic conditions and suggests that the same electron transport pathway can be used for more than one electron acceptor.

Shewanella oneidensis MR-1 is a facultative, gram-negative, nonfermenting γ-proteobacterium whose respiratory versatility has prompted interest in its use in bioremediation. MR-1 can reduce a variety of compounds, including iron(III), manganese(IV), nitrate, nitrite, thiosulfate, sulfite, trimethylamine *N*-oxide (TMAO), dimethyl sulfoxide (DMSO), fumarate, uranium(VI), technetium(VII), chromium(VI), elemental sulfur, and carbon tetrachloride (8, 9, 11, 13, 14–16, 19, 20, 22, 23, 30). Uranium and chromium are common groundwater pollutants, due to their use in defense and industrial applications (6, 31). MR-1 may be useful for in situ immobilization of uranium and chromium in subsurface environments because of its ability to reduce toxic and soluble hexavalent uranyl (UO_2^{2+}) and chromate (CrO_4^{2-}) to less soluble and less toxic forms [U(IV) and Cr(III)].

Despite the interest in U(VI) and Cr(VI) reductive immobilization, little is known about the mechanism of U(VI) and Cr(VI) reduction in MR-1 or in metal-reducing bacteria in general. An in vitro study (21) localized the chromate reductase activity of MR-1 cells growing on fumarate to the cytoplasmic membrane (CM). Other researchers reported that a MR-1 mutant lacking the cytochrome *c*552 nitrite reductase (SO3980) is deficient in its ability to reduce both nitrite and uranyl (39; J. R. Dale, R. Wade, and T. J. DiChristina, Abstr. 103rd ASM Gen. Meet. Am. Soc. Microbiol., abstr. Q389, 2003).

There are significant differences in the uptake and reduction of Cr(VI) and U(VI) by MR-1. Because of its similarity in structure to sulfate, it was shown that the chromate anion was taken up by cells through sulfate uptake channels in *Pseudomonas fluorescens* (29), and the same could be true for MR-1. Reduced chromium [Cr(III)] precipitates both inside the cytoplasm as round globules and on the outside of MR-1 cells (19). In contrast, there is no evidence that the uranyl cation is taken up inside the cytoplasm. In the case of *Shewanella putrefaciens* strain CN32, there is extensive precipitation of U(IV) as uraninite in the periplasm and outside the cells (3) and the same is observed for MR-1. Additionally, when H_2 is used as an electron donor, MR-1 cells are able to grow with U(VI) as the sole terminal electron acceptor, but there have been no reports of growth on Cr(VI).

Gene expression studies have the potential to help elucidate the mechanism of U(VI) and Cr(VI) reduction and evaluate how bacterial cells react to the presence of these toxic compounds. The sequencing of the MR-1 genome by The Institute for Genomic Research makes MR-1 a prime candidate for gene expression studies of U(VI) and Cr(VI) reduction. The goal of this investigation was to identify those genes upregulated during the reduction of U(VI) and Cr(VI) that may be part of the reduction pathway(s), as well as to gain an insight into the physiological changes undergone by cells actively

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reducing U(VI) and Cr(VI). The roles of some of the genes identified were further investigated by using appropriate mutants. The remaining genes, for which no mutants were available, are good candidates for further detailed study. This work is the first to evaluate the changes in mRNA expression profiles in MR-1 during anaerobic U(VI) and Cr(VI) reduction using DNA microarrays that include the approximately 5,000 putative open reading frames in MR-1's genome (4), and it provides essential information to pursue future hypothesis-driven studies.

MATERIALS AND METHODS

Bacterial cultures. *S. oneidensis* MR-1 was obtained from Oak Ridge National Laboratory and stored as freezer stocks at −80°C until each use, when the culture was maintained aerobically on Luria-Bertani (LB) agar (tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 10 g/liter; agar, 12 g/liter).

MR-1 cells were grown in a minimal medium we designated *Shewanella* medium (SM). One liter of this medium contains 0.68 g of NaCl, 0.285 g of $MgCl_2 \cdot 6H_2O$, 56.5 mg of $CaCl_2 \cdot 2H_2O$, 0.3975 g of Na_2SO_4, 0.15 g of NH_4Cl, 12.5 mg of $Na_2HPO_4 \cdot 12H_2O$, 0.3 g of KCl, and 3.5 g of HEPES. All chemicals were obtained from Sigma (St. Louis, MO). Trace elements and vitamins were added according to the recipe of Widdel and Bak (42). The pH of the medium was adjusted to 7.2. In all experiments, the electron donor was lactate (30 mM), and bicarbonate (30 mM) was also added. In some of the experiments, SM was amended with 10 ml of LB medium per liter.

Microarray experiments. The initial part of all microarray experiments, up to but excluding TRIzol addition, were conducted in a Coy anaerobic chamber containing an N_2 atmosphere with 1.5 to 2% H_2. Anaerobic sterile SM medium (400 ml in a Wheaton flask) was inoculated with 8 ml of strain MR-1 grown aerobically overnight in LB. Nitrate (as sodium nitrate; Sigma) served as the electron acceptor and was added at a concentration of 1.5 mM sequentially every 12 h to avoid the accumulation of toxic levels of nitrite. It should be noted that although nitrate was used to grow the cells for the microarray experiments, nitrate and nitrite were not present in solution during the final experiment in which cells to be exposed to Cr(VI) or U(VI) were harvested. Nitrate and nitrite were determined by ion chromatography using an LC 20 ion chromatograph (Dionex, Sunnyvale, CA) equipped with a CD 25 detector and an IonPac AS11-HC (4-mm) column (Dionex) with a sodium hydroxide buffer (25 mM NaOH) at a flow rate of 1.5 ml/min. After a total of 6 mM nitrate was added, the cells were transferred to 500-ml sterile centrifuge bottles and centrifuged for 15 min at 5,500 × *g* at 4°C. The pellet was resuspended in 400 ml of sterile fresh medium, and again, a total of 6 mM nitrate was added as sequential 1.5 mM additions. Cells were centrifuged, and the pellet was resuspended in 400 ml of fresh medium a second time. The procedure was repeated a third time, except nitrate was added to a total concentration of 2.5 mM. When nitrate and nitrite concentrations dropped below the detection limit of 5 μM, the optical density at 600 nm was 0.512. Approximately 1 h later, 10 ml of cells was aliquoted into 250-ml Wheaton flasks in the anaerobic chamber. Triplicate bottles were spiked with 0.1 mM Cr(VI) in the form of potassium chromate (Sigma) from a sterile 2.6 mM anaerobic stock and 0.1 mM U(VI) in the form of uranyl acetate (Electron Microscopy Science, Fort Washington, PA) from a sterile 10 mM anaerobic stock. Cells were harvested at 14 and 34 min for Cr(VI) (corresponding to 57 and 99% reduction) and at 6 and 11 h for U(VI) (corresponding to 21 and 52% reduction). The choice of sampling times was based on the kinetics of reduction, which are very different for Cr(VI) and U(VI). Samples were filtered through a 0.20-μm filter; Cr(VI) or U(VI) remaining in solution were measured colorimetrically with diphenyl carbazide (38) and fluorometrically with a Fluorolog 3 (Jobin Yvon, Inc.), respectively. The control culture consisted of cells maintained without added electron acceptor to avoid changes (e.g., growth) over the large range of sampling times for both metals. The control was sampled at 10 min, 3 h, and 6 h. Using any specific electron acceptor as a control would have had the double disadvantage of (i) potentially overshadowing the upregulation of genes involved in the reduction of both the electron (e^-) acceptor and either U(VI) or Cr(VI) and (ii) supporting growth, which neither U(VI) nor Cr(VI) does under these experimental conditions. The choice not to add an e^- acceptor to the control culture was intended to minimize both these effects.

Cells were harvested by centrifuging 1.5 ml of culture in Eppendorf tubes in a microcentrifuge in the anaerobic chamber. After discarding the supernatant outside of the anaerobic chamber, 1 ml of TRIzol reagent (Gibco BRL, Gaithersburg, MD) was immediately added. The pellet was resuspended and samples were frozen immediately at −80°C until RNA extraction. Total cellular RNA was isolated using TRIzol Reagent according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Ambion, Inc., Austin, TX) to digest any residual chromosomal DNA and then purified with the RNeasy kit (QIAGEN, Chatsworth, CA). The concentration and purity of dilutions of the RNA were checked with a UV spectrophotometer at wavelengths of 260 nm (in water) and 280 nm (in 10 mM Tris, pH 7.5), respectively. Concentrations of RNA were approximately 1,000 μg/ml. RNA from triplicates was pooled prior to cDNA synthesis. RNA from the three time points of the control culture was combined in a ratio of 2:3:1 and used as a control for all hybridizations. The advantage of using this approach over using a control obtained at the same time as the sample is that we could discern the trend in expression over time because the two time points are hybridized to a common background. Using a mixture of time points heavily skewed towards short equilibration times (5:1) is a compromise that permits the use of the same control for U and Cr, despite their differences in reduction kinetics.

Approximately equal amounts of total cellular RNA from control and experimental conditions (10 to 15 μg each) were used as templates to make probe cDNA. The total cellular RNA for each reaction mixture (10.5 μl) was mixed with 1.5 μl of random hexamers (3 μg/μl) (Gibco BRL), incubated at 70°C for 10 min, and immediately transferred to ice. The following were then added: 4 μl 5× first-strand buffer, 2 μl 0.1 M dithiothreitol, 1 μl of deoxynucleoside triphosphate mixture (10 mM dATP, dCTP, dGTP, and 0.4 mM dTTP), and 1.2 μl of 1 mM fluorophor Cy3-dUTP (control) or Cy5-dUTP (experimental) (Amersham Biosciences, Piscataway, NJ). The mixture was incubated at room temperature for 10 min in the dark. A total of 1 μl Superscript reverse transcriptase (Gibco BRL) was added, and the mixture was incubated at 42°C for 90 min. cDNA was purified with Qiaquick PCR purification kits (QIAGEN) and concentrated with a Speedvac (ThermoSavant SPD111V SPD) centrifuge by spinning 20 μl of the mixture for about 1 h down to a final volume of 5 μl.

cDNA was hybridized onto microarray slides printed at Oak Ridge National Laboratory. The array design and construction were described previously (4). Cy3- and Cy5-labeled cDNA was combined and brought to 15.4 μl with filtered Tris-EDTA buffer. The following were added to the cDNA: 1.48 μl of tRNA ($10 \text{ mg} \cdot \text{ml}^{-1}$), 4.18 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 mM sodium citrate), and 2.96 μl of 2% sodium dodecyl sulfate for a total of 24 μl. The mixture was heated at 98°C for 2 min and then cooled for 2 min at room temperature. The mixture was placed on a 22- by 40-cm coverslip, and the slide was placed over the coverslip, array side down. Slides were placed in a waterproof CMT slide chamber (Corning, Corning, NY) with 50 μl of filtered Tris-EDTA dotted beside the coverslip to maintain moisture. The chambers were submerged in a water bath at 65°C for 7 h in the dark. Following hybridization, slides were washed with 1× SSC–0.03% sodium dodecyl sulfate (1 min), 0.2× SSC (1 min), and finally 0.05× SSC (2 min); dried in microtiter plate carriers in a centrifuge for 2 min at 28 × *g*; and then immediately scanned with a GenePix 4000B Scanner (Axon Instruments, Union City, CA).

GenePix Pro 3.0 software (Axon Instruments) was used for image analysis to determine fluorescence image intensity and background intensity, as well as to identify spots of poor quality. GenePix computes a log ratio data set. This log ratio is a base two logarithm of the ratio of the medians of the two wavelengths (635 nm/532 nm or Cy5/Cy3 or red/green). Only spots where the mean fluorescence within the feature pixel was >2 standard deviations above the background for both wavelengths were used for data analysis.

The computer program Microsoft Access was used for statistical analysis and normalization of microarray data. The normalization method used is based on the premise that most genes on the array will not be differentially expressed and therefore the arithmetic mean of the ratios from every feature on a given array should be equal to 1. If the mean is not 1, a value is computed which represents the amount by which the ratio data should be scaled such that the mean value returns to 1. This value is the normalization factor. The normalization factor is added to all of the spots so that the mean of the ratios is 1 (1). This allows for better comparison between slides.

Since the entire genome of MR-1 was spotted twice on each slide, one copy on the upper half of the slide and one on the lower half; two separate data sets were obtained per slide. For each time point, two slides were successfully hybridized, allowing the averaging of up to four log ratio values for each gene. Average values of the log ratio values and standard deviations were computed for each gene. Genesifter software (VizX Lab LLC, Seattle, WA) was used to produce color-coded graphs known as heat plots for all the genes upregulated ≥3 fold for either metal or the genes upregulated or downregulated under both Cr(VI)- and U(VI)-reducing conditions.

Mutant characterization. Mutants defective in the functions of specific genes and kindly made available by other researchers studying MR-1 (Table 1) were

TABLE 1. Mutants tested for Cr(VI) and U(VI) reduction[a]

Organism	Gene(s) knocked-out	SO no. of knocked-out gene	Description	Function of knocked-out gene	Source	Reference
MR-1			Lake Oneida isolate		K Nealson	22
MR-1R			Spontaneous rifampin mutant of WT MR-1		D. Saffarini	2
SR-8	*mtrA* *mtrB* *mtrC*	SO1778 SO1777 SO1776	Transposon mutant of MR-1R defective in Fe(III) and Mn(IV) reduction; *mtrC*::Tn*5* Rif^r Kan^r $MtrC^-$ $MtrA^-$ $MtrB^-$	All three genes are involved in Fe(III) and Mn(IV) reduction	D. Saffarini	2
SR-522	*mtrC*	SO1778	MR-1R *mtrC*::Tn*5* Kan^r transformed with PRK415 carrying *mtrAB* downstream of a *lac* promoter; $MtrC^-$ $MtrA^+$ $MtrB^+$ Tc^-	Outer membrane decaheme involved in Fe(III) reduction	D. Saffarini	2
SR-A11=SR-524	*mtrA*	SO1777	MR-1R *mtrA*::pVIK165 Rif^r Kan^r transformed with pRK415 carrying *mtrB* downstream of a *lac* promoter; $MtrC^+$ $MtrA^-$ $MtrB^+$ Tc^r	Periplasmic decaheme involved in Fe(III) and Mn(IV) reduction	D. Saffarini	2
AQ38	*mtrB*	SO1776	Transposon mutant of MR-1 defective in metal (Fe and Mn) reduction; *mtrB*::Tn*phoA′* Kan^r	Outer membrane protein involved in Fe(III) and Mn(IV) reduction	D. Newman	27
SR-515	*fccA*	SO0970	Transposon mutant of MR-1R defective in fumarate reduction; MR-1R *fccA*::Tn*5*; *fccA* Kan^r	Periplasmic flavocytochrome *c* fumarate reductase	D. Saffarini	33
H2	*menC*	SO4575	Transposon mutant of MR-1 defective in menaquinone production; *menC*::mini-Tn*10 nptII* Kan^r	Precursor of menaquinone	D. Newman	36
MR1-CYMA	*cymA*	SO4591	Gene replacement mutant of MR-1; *cymA*::Kan^r	Periplasmic tetraheme cytochrome involved in fumarate, Fe(III), and Mn(IV) reduction	C. Myers	26

[a] Kan, kanamycin; Tc, tetracycline.

tested for Cr(VI) and U(VI) reduction. The mutants and the wild type (WT) were grown from frozen stock on LB plates with kanamycin (Kan) (50 $\mu g \cdot ml^{-1}$) when appropriate. An overnight liquid culture was grown aerobically from a single colony into SM with Kan (10 $\mu g \cdot ml^{-1}$) where appropriate. The culture was used as an inoculum for serum bottles containing sterile anaerobic SM with 5 mM TMAO with or without Kan. TMAO was used as an e^- acceptor because it was one of the few electron acceptors on which all the mutants could grow. After overnight (12-h) growth on TMAO, the cells were harvested and washed three times with SM using centrifugation-resuspension cycles. For the WT and each mutant, the cell pellet was resuspended in 0.5 ml of sterile anaerobic SM, and the cell culture was added to each of duplicate serum bottles [with 25 ml medium for Cr(VI) and 50 ml for U(VI)] to a final optical density at 600 nm of 0.1, thus insuring that mutants and the WT were present at the same concentration. The serum bottles contained SM (and 10 $\mu g \cdot ml^{-1}$ Kan and 0.5 mM IPTG [isopropyl-β-D-thiogalactopyranoside] in some cases) with either 0.1 mM Cr(VI) as sodium chromate, 0.1 mM U(VI) as uranyl acetate, or 5 mM Fe(III) citrate. In addition, one serum bottle of the WT was autoclaved and served as a heat-treated control. The integrity of the cells is not compromised when they are autoclaved in serum bottles. Over time, the bottles were sampled anaerobically and analyzed for solution U(VI), Cr(VI), or Fe(II). U(VI) and Cr(VI) were measured as described above, and Fe(II) was measured colorimetrically with ferrozine (37). Fe(II) was measured for the sole purpose of confirming the phenotypes of mutants lacking the ability to reduce Fe(III) citrate.

RESULTS AND DISCUSSION

Gene expression studies have the potential to help elucidate the mechanisms by which bacteria reduce metals. Though upregulation of genes alone is not equivalent to involvement in Cr(VI) or U(VI) reduction, microarray data help to hone in on genes worthy of further study. The results of these microarray experiments are shown in the form of heat plots (color-coded plots) that display genes upregulated ≥3-fold during Cr(VI) or U(VI) reduction (see Tables S1 and S2 in the supplemental data) or both (Table 2). The threefold criterion was chosen for this analysis as a conservative indication of upregulation. For U(VI), 121 genes fell into this category for either time point, whereas for Cr(VI), 83 genes were upregulated ≥3-fold (see Tables S1 and S2 in the supplemental data).

Table 2 shows genes upregulated and downregulated under both U(VI)- and Cr(VI)-reducing conditions (≥3-fold). The design of the experiment (using the same reference for all experiments) allows the quantitative comparison of the expression level of genes during the reduction of both metals. Of 32 genes upregulated for both metals, 10 were hypothetical proteins and 12 were cytochromes. The same cluster of three genes (SO4483 to SO4485, referred to as the SO4483-5 cluster) corresponds to the most upregulated genes for both metals. The genes downregulated for both metals were primarily associated with regulation, energy production, and transcription/translation.

Genes upregulated during Cr(VI) and U(VI) reduction were grouped into categories according to their annotated function

TABLE 2. Genes upregulated and downregulated for both Cr(VI) and U(VI)[a]

Gene no.	Cr_t1 Cr_t2 U_t1 U_t2	Genes	Cr_t1	Cr_ t2	U_t1	U_t2	Function
1		SO4484 cytochrome c-type protein Shp	12.1	16.0	32.0	23.4	cyt
2		SO4483 cytochrome b, putative	12.9	8.8	9.8	14.4	cyt
3		SO4485 diheme cytochrome c	7.7	11.4	8.0	12.1	cyt
4		SO4476 spheroplast protein y precursor, putative	3.3	7.3	4.6	9.3	me
5		SO0970 fumarate reductase flavoprotein subunit	5.6	4.0	3.4	10.0	cyt
6		SO2007 conserved hypothetical protein	1.0	6.8	5.7	7.9	hyp
7		SO3718 thiol:disulfide interchange protein, DsbA family	5.6	8.0	4.2	1.0	me
8		SO3286 cytochrome d ubiquinol oxidase, subunit I (cydA)	3.8	4.4	6.0	3.9	cyt
9		SO1111 bacterioferritin subunit 2 (bfr2)	4.8	4.9	5.1	2.8	cyt
10		SO3842 conserved hypothetical protein	1.0	4.0	7.0	5.4	hyp
11		SO1776 outer membrane protein precursor MtrB (mtrB)	2.3	5.5	3.1	5.5	me
12		SO1357 ribosomal protein S16 (rpsP)	1.0	3.7	2.1	9.4	tr
13		SO1428 outer membrane protein	2.0	1.0	2.3	10.6	me
14		SO1337 hypothetical protein	1.0	3.2	7.5	3.7	hyp
15		SO1429 anaerobic dimethyl sulfoxide reductase, A subunit (dmsA-1)	2.3	2.0	3.8	6.3	cyt
16		SO1777 decaheme cytochrome c MtrA (mtrA)	2.5	4.5	4.0	3.0	cyt
17		SO1126 chaperone protein DnaJ (dnaJ)	1.0	4.1	2.1	6.4	str
18		SO3417 peptidyl-prolyl cis-trans isomerase SlyD (slyD)	1.0	4.0	5.3	3.3	str
19		SO2016 heat shock protein HtpG (htpG)	1.0	3.7	3.0	5.8	str
20		SO3765 conserved hypothetical protein	1.0	3.1	3.5	4.3	hyp
21		SO4520 oxygen-independent coproporphyrinogen II	1.0	3.1	5.7	2.1	cyt
22		SO1778 decaheme cytochrome c (omcB) or (mtrC)	2.4	2.6	4.1	2.7	cyt
23		SO1274 conserved hypothetical protein	3.7	1.0	5.1	1.6	hyp
24		SO1779 decaheme cytochrome c (omcA)	2.9	2.7	2.8	2.8	cyt
25		SO0662 conserved hypothetical protein	1.0	3.2	4.0	2.8	hyp
26		SO4649 conserved hypothetical protein	3.1	1.0	1.0	5.8	hyp
27		SO1430 anaerobic dimethyl sulfoxide reductase, B subunit (dmsB-1)	1.0	4.1	3.2	2.5	cyt
28		SO3285 cytochrome d ubiquinol oxidase, subunit II (cydB)	1.0	3.4	4.6	1.0	cyt
29		SO1112 bacterioferritin subunit 1 (bfr1)	1.0	2.7	3.4	2.8	cyt
30		SO3888 conserved hypothetical protein	1.0	4.7	1.0	3.1	hyp
31		SO3387 hypothetical protein	3.1	1.0	3.1	1.0	hyp
32		SO1427 hypothetical protein	1.0	1.0	4.0	1.7	hyp
33		SO3273 conserved hypothetical protein	1.0	0.3	1.0	0.3	hyp
34		SO1334 prolipoprotein diacylglyceryl transferase	1.0	0.3	0.2	0.3	nrg
35		SO1558 phosphate regulon response regulator Pho	0.2	0.3	0.3	1.0	tr
36		SO1493 4-alpha-glucanotransferase (malQ)	1.0	0.2	0.2	0.2	nrg
37		SO1522 L-lactate permease, putative	1.0	0.2	0.2	0.2	nrg
38		SOA0127 hypothetical protein	0.2	0.2	0.2	1.0	hyp
39		SO1557 outer membrane porin, putative	0.2	0.2	0.2	0.6	me
40		SO1723 phosphate ABC transporter, permease protein	0.1	0.2	0.2	0.3	nrg
41		SO1239 conserved hypothetical protein	0.1	0.1	0.2	0.1	hyp
42		SO1855 ribosome modulation factor (rmf)	0.3	1.0	0.1	0.4	tr
43		SO0858 sodium:alanine symporter family protein	0.3	0.5	0.1	0.2	tr
44		SO1238 acyltransferase family protein	0.1	0.2	0.3	0.3	tr

[a] Genes were included in this list either (i) because they were up- or downregulated three or more times for at least one time point for both metals or (ii) because they were contiguous to a gene that was upregulated three or more times. The two times points sampled for both U(VI) and Cr(VI) reduction are shown. The brighter the red color is, the greater the fold upregulation. Similarly, the brighter the green color is, the greater the fold downregulation. The functions listed are as follows: cyt, cytochrome and heme synthesis; hyp, hypothetical; sec, secretion; me, membrane; str, stress; tr, transcription and translation; mot, motility; nrg, energy production.

(Table 2; see Tables S1 and S2 in the supplemental data). The percentage of total genes in each category was quantitatively similar for U(VI) and Cr(VI), with the exception of detoxification genes, which were upregulated for Cr(VI) but not U(VI). Genes encoding hypothetical proteins were the largest group for both metals: 34% for U(VI) and 29% for Cr(VI). Two other categories of genes appear important based on the microarray data: genes encoding cytochromes (and other electron transport proteins) and stress proteins (both membrane and cytoplasmic). In this work, we focused on investigating genes involved in electron transport, although we include a brief discussion of results related to stress and detoxification proteins.

Genes encoding cytochromes and other electron transport proteins. A large fraction of the genes upregulated [16 and 20% for U(VI) and Cr(VI), respectively] correspond to those encoding putative cytochromes, cytochrome synthesis proteins, or noncytochrome reductases (Table 2). This is consistent with the major role of cytochromes in metal reduction (12). Most notably, three cytochrome genes, arranged in a cluster, were highly upregulated for both Cr(VI) and U(VI) reduction: SO4483, SO4484, and SO4485. Additionally, genes encoding proteins involved in the reduction of various electron acceptors were upregulated: fumarate reductase (*fccA*), the DMSO reductase cluster (*dmsAB-1*), cytochrome *d* ubiquinol oxidases (*cydAB*), and various genes involved in the reduction of Mn(IV) and soluble Fe(III), such as the *mtrCAB* cluster. We discuss these genes below.

(i) Genes involved in the reduction of Fe(III) and Mn(IV). Genes encoding some of the proteins involved in Fe(III) citrate and Mn(IV) reduction (*mtrA*, *mtrB*, *mtrC* [also known as *omcB*], and *omcA*) were upregulated during U(VI) and Cr(VI) reduction (Table 2). Fe(III) and Mn(IV) are insoluble in most environments and using them as electron acceptors requires the export of electrons to the outer membrane (OM). A recent review of Fe(III) and Mn(IV) reduction (13) describes the current model of the Fe(III) reduction pathway (originally posited in reference 2), which proposes that electrons are transferred from NADH dehydrogenase to the quinone pool, e.g., menaquinone (MQ), in the CM. The reduced MQ in turn reduces CymA, a periplasmic tetraheme cytochrome *c* anchored in the CM. CymA subsequently transfers electrons di-

rectly or indirectly (through a periplasmic cytochrome *c*3) to MtrA, a periplasmic decaheme cytochrome, which reduces another decaheme cytochrome, MtrC (OmcB), located in the OM. OmcA, also a decaheme OM protein, is involved in Mn(IV) reduction (25). The exact role of MtrB in Fe(III) reduction is not known. CymA is an efficient electron donor for the soluble fumarate reductase (SO0970 or *fccA*) (35). The absence of CymA in MR-1 results in a loss of the ability to respire with Fe(III), fumarate, and nitrate (26).

The role the genes involved in Fe(III) and Mn(IV) reduction play in U(VI) and Cr(VI) reduction was evaluated in studies of mutants (Table 1). Most the mutants tested showed hindered ability to reduce U(VI) and Cr(VI) relative to the WT (Fig. 1, 2, and 3), except the mutant deficient in CymA (Fig. 3B). A mutant deficient in MenC (a precursor of menaquinone) showed a reduced rate and extent of U(VI) and Cr(VI) reduction (Fig. 1B and 2), indicating the involvement of menaquinone (or a precursor to menaquinone synthesis downstream of MenC) in electron transport to U(VI) and Cr(VI) in MR-1. The menaquinone pathway yields at least one molecule capable of electron transfer to another environmentally relevant compound, carbon tetrachloride (40). CymA is the next protein in the nitrate, fumarate, and Fe(III) electron transport pathway. Yet, the absence of CymA has no effect on Cr(VI) reduction (Fig. 3B) and the least effect on U(VI) reduction of all the mutants considered that were deficient in Fe(III) reduction (Fig. 1). We thus conclude that CymA is not involved in Cr(VI) reduction. It is involved in U(VI) reduction, but it is not the only protein transferring electrons from menaquinone to downstream proteins because if that were the case, mutants deficient in MenC or CymA would have identical phenotypes. We propose that there exists an as-yet-unknown protein that plays the same role as CymA in the case of U(VI) reduction. The diminished role of CymA for U(VI) and Cr(VI) reduction is a significant difference with the reduction pathways of nitrate, fumarate, Fe(III), and Mn(IV), all of which require CymA.

For U(VI) reduction, the phenotype of the mutants deficient in MenC and MtrA have similar phenotypes (Fig. 1B), indicating that all the electrons passed downstream by MQ eventually reach MtrA. Mutants deficient in MtrA, MtrB, or MtrC showed virtually identical phenotypes with respect to U(VI) reduction, implying the involvement of all three genes as part of a pathway for U(VI) reduction and a flow of electrons from MtrA to MtrC. The fact that the mutant deficient in MtrB has the same phenotype as those lacking MtrA and MtrC suggests its involvement in U(VI) reduction. A separate set of MtrB and MtrC mutants (MTRB1) (24) and OMCB1 (24, 25) showed similar results for U(VI) reduction (C. R. Myers, personal communication).

For Cr(VI) reduction, MtrA- and MtrC-deficient mutants showed similar phenotypes (Fig. 2). MtrB and MenC mutants also approximately clustered together. The effect on Cr(VI) reduction is greater in a MtrA-deficient mutant than in a MenC-deficient one, suggesting there is an alternative source of electrons for MtrA other than MenC. This suggests the existence of another as-yet-unknown protein that transfers upstream electrons to MtrA, bypassing MenC. Finally, it is clear that MtrA is the only source of electrons for MtrC, as their phenotypes are almost identical.

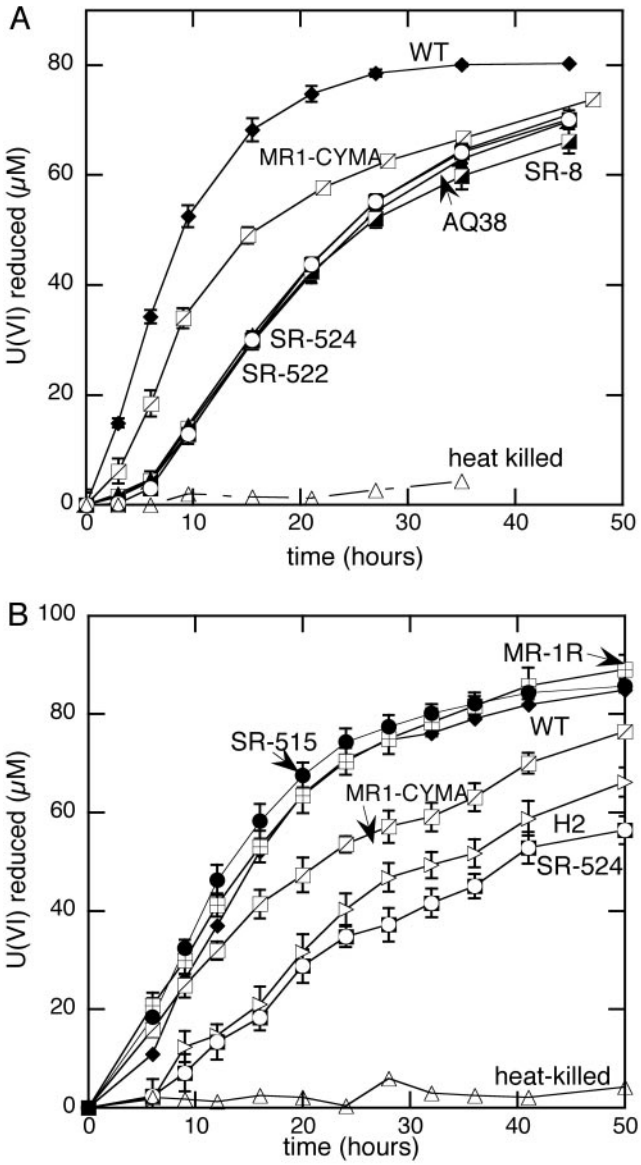

FIG. 1. U(VI) reduction by WT (MR-1) and MR-1 mutants deficient in various proteins. (A) MtrB (AQ38), MtrC (SR-522), MtrA (SR-524), MtrCAB (SR-8), and CymA (MR1-CYMA). (B) Fumarate reductase FccA (SR515), CymA (MR1-CYMA), the menaquinone precursor MenC (H2), and MtrA (SR-524). Error bars represent the data range for duplicate cultures.

In summary, MenC, CymA, and MtrCAB are involved in U(VI) reduction and there is probably another protein transferring electrons from MenC to MtrA. For Cr(VI), MenC and MtrCAB are involved along with a second unknown protein that transfers electrons from upstream of MenC to MtrA. CymA is not involved in Cr(VI) reduction.

It is noteworthy that U(VI)- and Cr(VI)- reducing activity was not abolished for any of the mutants considered. A likely explanation for this finding is that there are multiple pathways for the reduction of these metals. Researchers (J. R. Dale, R. Wade, and T. J. DiChristina, Abstr. 103rd ASM Gen. Meet., abstr. Q389, 2003) have shown that knocking out the nitrite

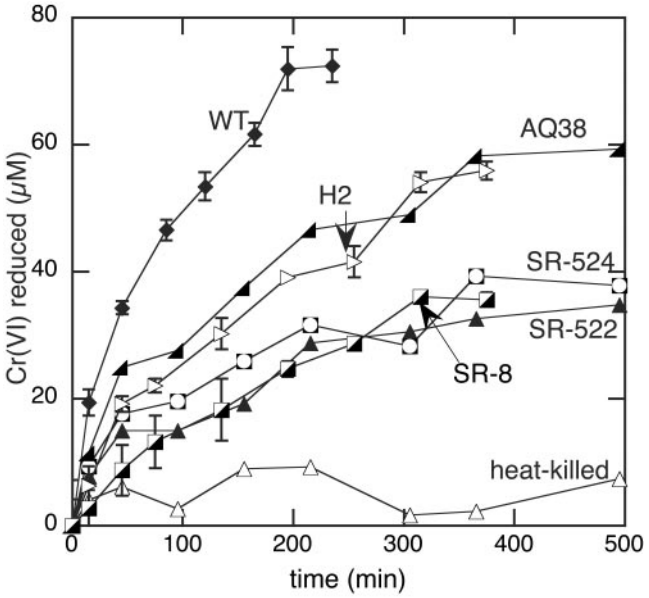

FIG. 2. Cr(VI) reduction by WT (MR-1) and MR-1 mutants deficient in MtrB (AQ38), MenC (H2), MtrA (SR-524), MtrC (SR-522), or MtrCAB (SR-8). Error bars represent the data range for duplicate cultures.

reductase (SO3980) in MR-1 resulted in abolishment of the U(VI)-reducing activity, suggesting it may be the terminal reductase for U(VI). In addition, other genes upregulated during Cr(VI) and U(VI) reduction and for which no mutants were tested (SO4483-5, discussed below, or genes encoding hypothetical proteins) may also be involved.

(ii) SO4483-5 cluster. The three genes in the highly upregulated cluster SO4483-5 are annotated as cytochromes and share homology with cytochromes from *Rhodobacter sphaeroides*. In *R. sphaeroides*, a novel cytochrome *c* sphaeroides heme protein (SHP) with a redox potential of −22 mV (18) is associated with a soluble diheme cytochrome *c* (DHC) with a lower redox potential (−254 mV) (18) that is thought to be the electron donor for SHP (10). A third cytochrome *b* of unknown redox potential is associated with the previous two (10). In MR-1, SO4484 is homologous to SHP (44.9% identity, 63.3% similarity); SO4485 is homologous to DHC (44% identity, 61% similarity) with a hydrophobic N-terminal sequence. This suggests that the protein encoded by SO4485 in MR-1 is a membrane-anchored periplasmic protein rather than a soluble protein, as is DHC in *R. spharoides*. SO4483 is similar to the *R. sphaeroides* cytochrome *b* associated with SHP (10). The hydrophobicity profile of the protein encoded by SO4483 is consistent with a membrane-spanning protein. These three genes were also highly upregulated during anaerobic growth on nitrate or TMAO (A. Beliaev, personal communication). The extent of upregulation of the SO4483-5 gene cluster for both U(VI) and Cr(VI) (as well as nitrate and TMAO) and the homology to an electron transport system in *R. sphaeroides* make it an excellent candidate for further study.

(iii) Fumarate and DMSO reductases. Genes encoding these reductases were found to be upregulated in the microarray results. However, mutants lacking the corresponding proteins did not show an effect on U(VI) or Cr(VI) reduction. A recent study (17) shows there is a sole physiological fumarate reductase in MR-1: FccA, encoded by SO0970. Despite consistent upregulation of SO0970 under all time points for both Cr(VI) and U(VI) (Table 2), there was no evidence of a difference between the reduction of U(VI) or Cr(VI) by a transposon mutant of FccA and by the WT (Fig. 1B and 3A). That result clearly shows that the fumarate reductase FccA is not involved in the reduction of the two metals under these conditions.

MR-1 has two protein complexes homologous to two of the three subunits of the *E. coli* trimeric anaerobic DMSO reductase complex (DmsABC) (34), DmsAB-1 and DmsAB-2. In the

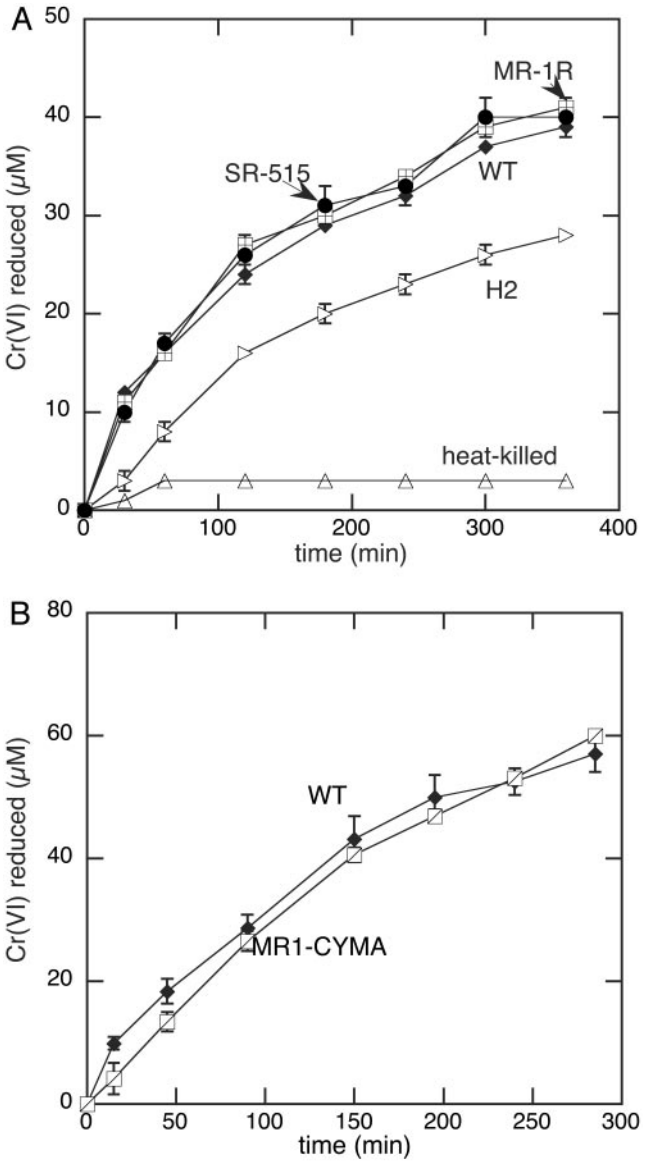

FIG. 3. Cr(VI) reduction by WT (MR-1), spontaneous rifampin mutant MR-1R, and MR-1 mutants deficient in various proteins. (A) Fumarate reductase (SR-515), CymA (MR1-CYMA), the menaquinone precursor MenC (H2), and MtrA (SR-524). (B) CymA (MR1-CYMA). Error bars represent the data range for duplicate cultures.

MR-1 genome annotation on the The Institute for Genomic Research website, the gene encoding the A subunit in both pairs (SO1429 and SO4358) appears to be incorrectly annotated as *dmaA* (it should be *dmsA*). In MR-1, *dmsA-1* (SO1429) and *dmsB-1* (SO1430) were upregulated to a greater extent for U(VI) than for Cr(VI). Genes on both sides of *dmsAB* (SO1427, SO1428 and SO1431) are significantly upregulated for U(VI) but not Cr(VI). SO1427 and SO1428 encode proteins with sequence homology to MtrA (75% similarity) and MtrB (58% similarity), respectively, and SO1431 is a hypothetical protein. Recent evaluation of U(VI) reduction by deletion mutants of *dmsA-1* and SO1427 showed no difference compared to the wild type (D. Saffarini and A. Beliaev, personal communication).

Therefore, for both DMSO and fumarate reductases, the expression data do not match the results of mutant data. We conclude that the fumarate reductase does not play a role in U(VI) or Cr(VI) reduction and that the DMSO reductase does not play a role in U(VI) reduction [mutants were not tested for Cr(VI)]. The upregulation of both the fumarate and DMSO reductases may be a nonspecific response to the redox potential established by the presence of Cr(VI) or U(VI) under nitrate-free anaerobic conditions rather than a specific response to the metals.

Genes associated with membrane and general stress. Approximately 11% and 6%, respectively, of the genes upregulated during U(VI) and Cr(VI) reduction encode putative cytoplasmic heat shock or environmental stress response proteins. Moreover, 7 and 6%, respectively, of the genes upregulated for U(VI) and Cr(VI) were annotated as encoding proteins involved in maintaining the structural integrity of the membrane(s) in response to stress or otherwise. We have intentionally classified the genes encoding proteins involved in membrane/periplasmic stress response separately from those associated with cytoplasmic stress to show the disproportionate cytoplasmic stress response to U(VI) but not to Cr(VI). This is an interesting finding because to date there is no evidence that U(VI) enters the cytoplasm. If it is the case, the presumed lack of a U(VI)-specific detoxification system may provide an opportunity for cytoplasmic damage by U(VI) and a concomitant stress response. In contrast, for chromium, there is evidence of active detoxification, consistent with less cytoplasmic stress (see "Detoxification," below).

Cytoplasmic stress proteins are typically classified as heat shock proteins, even though their function extends to other forms of environmental stress such as the presence of heavy metals (32). The cytoplasmic stress proteins upregulated under both U(VI)- and Cr(VI)-reducing conditions are chaperones homologous to GroEL, SlyD, DnaJ, and HtpG, all proteins whose function is to aid in protein folding and promote refolding of polypeptides generated under stress. A study investigating the heat shock response of the gram negative bacterium *Mycoplasma pneumoniae* (41) showed striking similarity with the transcription profile under U(VI)-reducing conditions. A large number of ribosomal proteins (e.g., L2, L3, S29, and S20) were upregulated along with heat shock chaperones (e.g., GroES) and ATP-dependent proteases (e.g., ClpP) (41). Similarly, U(VI) reduction caused the upregulation of chaperones (e.g., ClpB and DnaJ), proteases (e.g., HtrA and HslV), ribosomal proteins (S16, S14, L23, L10, L28, L16, and S1), and isomerases (PpiD and SlyD) (see Table S2 in the supplemental data). With the exception of the ribosomal proteins, all proteins are usually associated with a stress response.

Several genes (e.g., SO4476 and SO3718) involved in membrane synthesis, as well as preserving membrane integrity, were upregulated (Table 2). The spheroplast protein y (Spy) is expressed abundantly in *Escherichia coli* when spheroplasts are formed (5). The upregulation of the gene encoding this protein (SO4476) during both U(VI) and Cr(VI) reduction may indicate stress due to the extensive accumulation of U(IV) and Cr(III) precipitates outside the outer membrane.

Genes (SO1808-09) annotated as *pspB* and *pspC* are upregulated under U(VI)- but not Cr(VI)-reducing conditions. The corresponding genes in *E. coli* are associated with cytoplasmic membrane stresses (7). A response to cytoplasmic membrane stress under U(VI)- but not Cr(VI)-reducing conditions is consistent with the accumulation of solid-phase U(IV) but not Cr(III) in the periplasm of MR-1.

Detoxification. A significant difference between the genes expressed under U(VI)- and Cr(VI)-reducing conditions was the upregulation of efflux pumps in the presence of Cr(VI). Genes SO0518-520, encoding putative efflux pumps, were highly upregulated in the presence of Cr(VI) at both time points, indicating a response to toxicity.

SO0520 is annotated as encoding a heavy metal efflux pump of the CzcA family. The membrane-bound CzcCBA protein complex mediates heavy metal resistance in *Alcaligenes eutrophus* by an active cation efflux mechanism driven by cation-proton antiport (28). The CzcA protein alone is able to mediate weak resistance to zinc and cobalt and is thus thought to be the central antiporter subunit. Our previous work has shown that the reduction of Cr(VI) inhibits growth in MR-1 and produces Cr(III), which forms globules in the cytoplasm and amorphous precipitates extracellularly (19). It is unknown whether a sufficient fraction of the Cr(III) formed in the cytoplasm remains in soluble form to trigger the upregulation of efflux pumps, if they are upregulated as a general response to Cr toxicity, or both. It is noteworthy that genes encoding cation efflux pumps are upregulated but that a gene (SO0986) encoding a putative chromate transporter is not.

These studies of gene expression and mutant phenotypes have helped elucidate the mechanism of U(VI) and Cr(VI) reduction by MR-1 and have shown a link between U(VI)/Cr(VI) reduction and that of Fe(III) citrate: the only upregulated genes whose involvement in U(VI)/Cr(VI) reduction was confirmed by mutant analyses were from the Fe(III) reduction pathway. However, the fact that reduction was not abolished by any of the mutants tested indicates that there are likely multiple pathways for U(VI) and Cr(VI) reduction in MR-1. A prime candidate for such an alternate pathway is the highly upregulated cluster SO4483-85, annotated as three cytochromes. Despite significant differences in the reduction of U(VI) and Cr(VI), such as the location of the reduced precipitates, great similarity was observed in which proteins were involved in the reduction of both metals. This study also gave insight into the physiological changes undergone by cells actively reducing U(VI) and Cr(VI). The reduction of these metals elicits a significant stress response, suggesting they may not be ideal electron acceptors for the cells. However, in sites where the metal concentration is lower than that considered in

this study, the stress response may be limited. For remediation purposes, an alternate electron acceptor may need to be provided to build enough biomass to accomplish consistent reduction.

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