Transcriptome and Proteome Dynamics of the Cellular Response of *Shewanella oneidensis* to Chromium Stress

S. D. Brown[1], K. Chourey[1], M. Thompson[2,3], N. C. VerBerkmoes[2,3], R. L. Hettich[2], S. Seal[4], S. Deshpande[4], J. Zhou[1], and D. K. Thompson[1] (PI)

[1]Environmental Sciences Division and [2]Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN; [3]Graduate School of Genome Science and Technology, University of Tennessee-Oak Ridge National Laboratory; [4]Advanced Materials Processing and Analysis Center, University of Central Florida

ABSTRACT

The overall goal of this DOE NABIR project is to characterize the molecular basis and regulation of hexavalent chromium [Cr(VI)] stress response and reduction by *Shewanella oneidensis* strain MR-1. Temporal genomic profiling and mass spectrometry-based proteomic analysis were employed to characterize the dynamic molecular response of *S. oneidensis* MR-1 to both acute and chronic Cr(VI) exposure. The acute stress response of aerobic, mid-exponential phase cells shocked to a final concentration of 1 mM potassium chromate (K_2CrO_4) was examined at post-exposure time intervals of 5, 30, 60, and 90 min relative to untreated cells. The transcriptome of mid-exponential cultures was also analyzed 30 min after shock doses of 0.3, 0.5, or 1 mM K_2CrO_4. The *tonB1-exbB1-exbD1* genes comprising the TonB1 iron transport system were some of the most highly induced coding sequences (CDSs) after 90 min (up to ~240 fold), followed by other genes involved in heme transport, sulfate transport, and sulfur assimilation pathways. In addition, transcript levels for CDSs with annotated functions in DNA repair (*dinP*, *recX*, *recA*, *recN*) and detoxification processes (*so3585*, *so3586*) were substantially increased in Cr(VI)-exposed cells compared to untreated cells. By contrast, genes predicted to encode hydrogenases (HydA, HydB), oxidoreductases (SO0902-03-04, SO1911), iron-sulfur cluster binding proteins (SO4404), decaheme cytochrome c proteins (MtrA, OmcA, OmcB), and a number of LysR or TetR family transcriptional regulators were some of the most highly repressed CDSs following the 90-min shock period. Transcriptome profiles generated from MR-1 cells adapted to 0.3 mM Cr(VI) differed significantly from those characterizing cells exposed to acute Cr(VI) stress without adaptation. Parallel proteomic characterization of soluble protein and membrane protein fractions extracted from Cr(VI)-shocked and Cr(VI)-adapted MR-1 cells was performed using multidimensional HPLC-ESI-MS/MS (both LCQ and LTQ instruments used). With LTQ, we were able to substantially increase proteome coverage by at least two-fold compared to LCQ analysis. These studies provide important insights into cellular chromium tolerance. Future research will focus on the structural and regulatory genes implicated in Cr(VI) reduction and detoxification.

METHODS

Bacterial Growth Conditions and Total RNA Isolation. For time-series microarray experiments, batch cultures of *S. oneidensis* MR-1 were grown to mid-exponential phase (OD_{600}, 0.5) under aerobic conditions in LB medium, followed by the addition of 2 M K_2CrO_4 to a final concentration of 1 mM. Untreated control cultures were grown in parallel with treated cultures. Control and treated cells were harvested for total cellular RNA extraction at 5, 30, 60, and 90 min post-K_2CrO_4 addition using the TRIzol reagent.

Microarray Data Analysis. Array hybridization signals were quantified using ImaGene version 5.5 (Biodiscovery, Inc.), followed by data transformation and normalization using the trimmed geometric mean method in GeneSite Light. ArrayStat™ (Imaging Research, Inc.) was used to determine the common error, remove outliers, and determine statistical significance. Genes exhibiting significant (p<0.05) changes in expression were further analyzed using the program Hierarchical Clustering Explorer version 3.0.

Chromate Reduction Assays and Metal Analysis. Residual chromate was measured using the 1,5-diphenylcarbazide method (Park et al., 2000) at 540 nm. The effect of the growth medium (LB) on potassium chromate was examined using a Varian (Cary-1E) UV-Visible Spectrophotometer. UV-Vis spectra were collected for K_2CrO_4 in LB at 5, 10, 30, 60, 90 min, and 24 h with reference to K_2CrO_4 in DI water. The surface chemistry of chromate in LB was characterized using a PHI 5400 X-ray photoelectron spectrometer at a base pressure of 10-10 Torr. Control samples of Cr^{+6} and Cr^{+3} were run as standards.

Proteomic Analysis. For all comparative studies, equal quantities of WT and Cr-treated cell lysates were digested with sequencing-grade trypsin, desalted, and concentrated. All samples were run in duplicate by LC/LC-ES-MS/MS on a quadrupole ion trap mass spectrometer (Thermo Finnigan LCQ) and a single time on a linear ion trap mass spectrometer (Thermo Finnigan LTQ). All datasets were searched with SEQUEST, filtered and sorted with DTASelect [minimum Xcorrs of 1.8 (+1), 2.5 (+2), 3.5 (+3)], and compared with Contrast. Differentially expressed proteins were extracted based on differences of 30% sequence coverage and/or four unique peptides between the WT and chromate-treated samples.

RESULTS

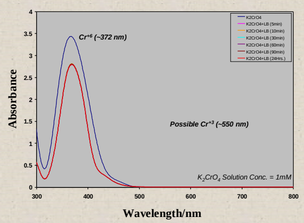


FIG 1. UV-Vis spectral analysis of potassium chromate (K_2CrO_4) in LB medium (no cells) over time.

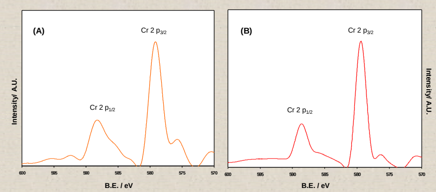


FIG 2. XPS spectra obtained for (A) K_2CrO_4 only and (B) K_2CrO_4 in LB medium for 24 h. In both cases, the oxidation state of Cr was +6. The amplitudes of the Cr (2p) peaks are comparable in both cases.

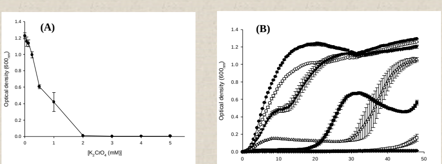


FIG 3. Dose-response growth curves describing the toxicity of potassium chromate (K_2CrO_4) for *S. oneidensis* MR-1. (A) The minimal inhibitory concentration (MIC) of K_2CrO_4 for MR-1 determined in LB broth under aerobic growth conditions at 30°C after 48 h. (B) Growth curves of MR-1 over 48 h in LB medium containing the following final concentrations (mM) of K_2CrO_4 were measured using a Bioscreen C reader: 0 (+); 0.06 (○); 0.125 (▼); 0.25 (Δ); 0.5 (■); 1 (□); and 2 (♦). The mean OD600 ± standard error (bars) for three replicate growth point measurements are shown and three independent experiments were conducted.

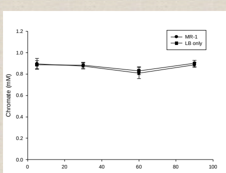


FIG 4. Chromate (K_2CrO_4) remaining in uninoculated LB medium and in mid-log phase, aerobic cultures of *S. oneidensis* MR-1.

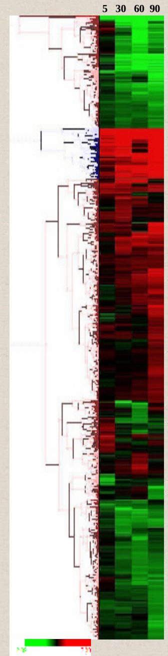


FIG. 5. Complete linkage clustering analysis of 910 *S. oneidensis* MR-1 genes exhibiting altered mRNA expression levels in response to 1 mM K_2CrO_4 exposure over time. Transcriptional profiles are shown at 5, 30, 60, and 90 min post-K_2CrO_4 shock. Individual genes are represented by a single row and each exposure time point is represented by a single column. Red represents the level of induction; green represents repression.

TABLE 1. Genes displaying high induction levels in temporal expression in response to potassium chromate stress.

		Mean (K_2CrO_4/control)[a]			
Gene	Gene Product t (min)[b]=	5	30	60	90
so3032	siderophore biosynthesis protein, putative	5	11	16	31
so3033	ferric alcaligin siderophore receptor	3	12	16	22
so3062	hypothetical protein	4	10	13	17
so3585	azoreductase, putative	6	61	28	30
so3586	glyoxalase family protein	4	26	16	13
so3587	hypothetical protein	4	18	10	14
so3667	conserved hypothetical protein	15	26	24	91
so3668	conserved hypothetical protein	12	15	16	46
so3669	heme transport protein	16	17	20	120
so3670	TonB1 protein	10	16	25	99
so3671	TonB system transport protein ExbB1	13	10	16	172
so3672	TonB system transport protein ExbD1	15	15	23	242
so3673	hemin ABC transporter, periplasmic hemin-binding protein	12	14	18	40
so3674	hemin ABC transporter, permease protein	14	15	17	34
so3675	hemin ABC transporter, ATP-binding protein	27	17	23	101
so3914	TonB-dependent receptor, putative	7	8	11	44

[a]Relative gene expression (fold induction) is presented as the mean ratio of the fluorescence intensity of K_2CrO_4-exposed cells to untreated control cells.

[b]Time in minutes at which cells were harvested for RNA isolation following addition of 1 mM K_2CrO_4 to the experimental culture.

TABLE 2. Protein Abundance Profiles for Highly Up-Regulated Genes Using LCQ vs. LTQ

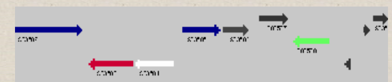
	45 min post exposure				90 min post exposure			
Gene	Con_LCQ	Con_LTQ	Cr_LCQ	Cr_LTQ	Con_LCQ	Con_LTQ	Cr_LCQ	Cr_LTQ
SO3032	6.5	8.5	9.3	22.3	0	5.7	14.8	31.4
SO3033	20.7	38	27.4	52.6	6.9	12.1	43.6	57.6
SO3062	0	0	0	0	0	0	0	0
SO3585	0	0	22.1	43.1	0	0	27.5	20.1
SO3586	0	0	0	38.4	0	0	27.5	60.1
SO3587	0	0	0	26.9	0	28.2	0	17.3
SO3667	10.3	23.8	83.2	96.8	0	0	69.7	91.9
SO3668	0	0	0	28	0	0	0	0
SO3669	3.4	30.7	67.7	74	0	9.3	71	71.6
SO3670	0	0	0	18.6	0	0	12	13.5
SO3671	0	18.1	18.1	18.1	0	0	18.1	20.4
SO3672	0	0	0	0	0	0	0	0
SO3673	0	9.5	57.1	65.3	0	0	62.8	63.1
SO3674	0	0	0	0	0	0	0	0
SO3675	0	24.3	40.2	62.5	0	0	53	72.9
SO3914	18.1	45.5	35.2	67.1	0	18.5	51.6	68.1

TABLE 3. Summary of Proteome Analysis

Condition	Instrument	No. Proteins Identified 1 pep*	No. Proteins Identified 2 pep[#]	Avg. Sequence Coverage (%)[†]
Control 1	LCQ	1318	894	28.66
45 min shock	LCQ	1238	816	31.77
Control 2	LCQ	1368	959	31.63
90 min shock	LCQ	1267	856	31.84
Control 1	LTQ	2294	1665	32.60
45 min shock	LTQ	2288	1723	33.28
Control 2	LTQ	2308	1751	33.14
90 min shock	LTQ	2291	1709	32.79
TOTAL		3015	2177	

LCQ: Thermo Finnigan ES-quadrupole ion trap
LTQ: Thermo Finnigan ES-linear ion trap

*Identified with at least 1 peptide per protein.
[#]Identified with at least 2 peptides per protein.
[†]Average sequence coverage per protein at the 2 peptide level.



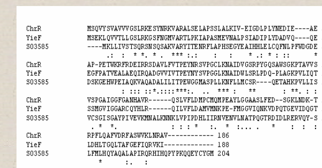
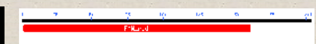


FIG. 6. Amino acid sequence alignment of *Pseudomonas putida* ChrR, *E. coli* YieF, and *S. oneidensis* SO3585. Asterisks indicate identical residues, colons indicate residues with a high level of similarity, and periods indicate residues with a lower level of similarity. The characteristic signature of the NADH_dh2 family of proteins is boxed. Clustal W alignment indicated that SO3585 shared approx. 28% sequence identity with ChrR and YieF, two soluble flavoproteins that possess chromate reductase activity (Ackerley et al., 2004; Park et al., 2000, 2002).

FIG. 7. Predicted 3D structure of SO3585



Putative conserved domain detected in SO3585 ➔NADPH-dependent FMN reductase

Azobenzene reductase from *Bacillus subtilis*.
E.C.1.-.-.- Oxidoreductases.
E.C.1.7.-.- Acting on other nitrogenous compounds as donors.
E.C.1.7.1.- With NAD(+) or NADP(+) as acceptor.

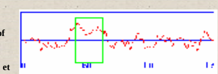
E.C.1.7.1.6 Azobenzene reductase.
Reaction: *N,N-dimethyl-1,4-phenylenediamine + aniline + NADP(+) = 4-(dimethylamino)azobenzene + nadph.*

Proteins from *Shewanella* species showing similarity to MR-1 So3585, So3586 and So3587 using BLASTP

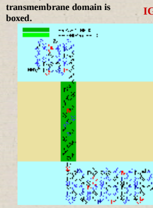
Genome	Protein sequence So3585	So3586	So3587
	Percent similarity, positives over region of similarity (ORNL gene designation)		
S. sp. PV-4	74, 155/207 (996)	85, 118/138 (995)	62, 97/155 (992)
S. amazonensis	63, 122/191 (653)	82, 114/138 (656)	59, 90/152 (657)
S. baltica OS155	89, 183/204 (268)	93, 129/138 (269)	90, 141/156 (271)
S. putrefaciens CN-32	89, 182/204 (3825)	93, 129/138 (3824)	89, 139/155 (3823)
S. oneidensis MR-1	100, 204/204 (3067)	100, 138/138 (3068)	100, 156/156 (3069)
S. denitrificans OS217T	N.D.*	81, 112/138 (1653)	71, 111/155 (2936)
S. frigidimarina ACAM 591T	N.D.*	79, 109/137 (2802)	66, 109/165 (2522)

*N.D. = Significant alignment not detected

Hydrophobicity plot of SO3587 (annotated hypothetical protein) using SOSUI (Mitaku et al., 2002). Predicted transmembrane domain is boxed.



IGIALIFADVSLYLAYFFVGLGV



MR-1 proteins SO3585 (azoreductase), SO3586 (glyoxalase family protein), and SO3587 (hypothetical protein) were all found in the LTQ data: (1) SO3585 in both the crude and membrane fractions, (2) SO3586 in both the crude and membrane fractions, and (3) SO3587 only in the membrane fraction.

CONCLUSIONS & FUTURE RESEARCH

- Global temporal alterations in the transcriptome and proteome of *S. oneidensis* MR-1 upon 1 mM potassium chromate exposure were determined in order to understand the cellular response to acute chromate stress. Genes and their corresponding protein products involved in iron and sulfate transport, cellular detoxification, and DNA repair were up-regulated in response to acute Cr(VI) exposure. Transcriptome profiles generated from cells adapted to 0.3 mM K_2CrO_4 (for 24 h) differed markedly from those characterizing cells exposed to acute Cr(VI) stress without adaptation (data not shown).
- Many of the proteins found to be up-regulated at the transcript level were found to have reproducible dramatic differences in % sequence coverage, # of unique peptides, and spectral count (all relative indicators of protein abundance). The new linear ion traps allow for a much greater detailed analysis of the proteome with ~2-3 times the proteome coverage allowing for comparisons of low abundance proteins not identified by conventional quadrupole ion traps. Replicate analyses of proteome samples with the linear ion trap are on-going.
- Chemical analysis indicated that Cr remains predominantly in the +6 oxidation state in LB medium (no cells) over the time course examined.
- MR-1 orthologs of known members of the LexA regulon (*recX*, *recA*, *recN*, and *dinP*) of *E. coli* were induced in response to Cr(VI) exposure.
- Molecular link between Cr exposure and iron/sulfate transport is being examined further.
- Deletion mutants defective in the structural gene so3585 and a DNA-binding response regulator have been created for strain MR-1 and are currently being characterized. This work has revealed other key gene targets for mutagenesis.
- We are in the process of investigating the possibility that SO3585, SO3586, and SO3587 form a protein complex anchored in the cytoplasmic membrane (by SO3587) that is involved in detoxification.

REFERENCES

Ackerley, D.F., C.F. Gonzalez, C.H. Park, R. Blake II, M. Keyhan, and A. Matin. 2004. Chromate-reducing properties of soluble flavoproteins from *Pseudomonas putida* and *Escherichia coli*. *Appl. Environ. Microbiol.* 70:873-882.

Mitaku, S., T. Hirokawa, and T. Tsuji. 2002. Amphiphilicity index of polar amino acids as an aid in the characterization of amino acid preference at membrane-water interfaces. *Bioinformatics* 18:608-616.

Park, C.H., C.F. Gonzalez, D.F. Ackerley, M. Keyhan, and A. Matin. 2002. Molecular engineering of soluble bacterial proteins with chromate reductase activity, p. 103-111. In R.E. Hinche et al. (ed.), Remediation and beneficial reuse of contaminated sediments. Batelle Press, Columbus, Ohio.

Park, C.H., M. Keyhan, B. Wielinga, S. Fendorf, and A. Matin. 2000. Purification to homogeneity and characterization of a novel *Pseudomonas putida* chromate reductase. *Appl. Environ. Microbiol.* 66:1788-1795.

ACKNOWLEDGMENTS

We thank Liyou Wu, Xueduan Liu, and Tingfen Yan for construction of whole-genome microarrays for *S. oneidensis* MR-1. We thank Xiu-Feng Wan for structure prediction of SO3585 and hydrophobicity plots, and Manesh Shah for proteome bioinformatic analyses. This research was supported by a grant from the U.S. Department of Energy Natural and Accelerated Bioremediation Research Program. Oak Ridge National Laboratory is managed by University of Tennessee-Battelle LLC for the Department of Energy under contract DOE-AC05-00OR22725.

OAK RIDGE NATIONAL LABORATORY