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Global Transcriptome Analysis of *Shewanella oneidensis* MR-1 Exposed to Different Terminal Electron Acceptors†

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To gain insight into the complex structure of the energy-generating networks in the dissimilatory metal reducer *Shewanella oneidensis* MR-1, global mRNA patterns were examined in cells exposed to a wide range of metal and non-metal electron acceptors. Gene expression patterns were similar irrespective of which metal ion was used as electron acceptor, with 60% of the differentially expressed genes showing similar induction or repression relative to fumarate-respiring conditions. Several groups of genes exhibited elevated expression levels in the presence of metals, including those encoding putative multidrug efflux transporters, detoxification proteins, extracytoplasmic sigma factors and PAS-domain regulators. Only one of the 42 predicted *c*-type cytochromes in MR-1, SO3300, displayed significantly elevated transcript levels across all metal-reducing conditions. Genes encoding decaheme cytochromes MtrC and MtrA that were previously linked to the reduction of different forms of Fe(III) and Mn(IV), exhibited only slight decreases in relative mRNA abundances under metal-reducing conditions. In contrast, specific transcriptome responses were displayed to individual non-metal electron acceptors resulting in the identification of unique groups of nitrate-, thiosulfate- and TMAO-induced genes including previously uncharacterized multi-cytochrome gene clusters. Collectively, the gene expression results reflect the fundamental differences between metal and non-metal respiratory pathways of *S. oneidensis* MR-1, where the coordinate induction of detoxification and stress response genes play a key role in adaptation of this organism under metal-reducing conditions. Moreover, the relative paucity and/or the constitutive nature of genes involved in electron transfer to metals is likely due to the low-specificity and the opportunistic nature of the metal-reducing electron transport pathways.

Metal ion reducing microbes play a central role in the biogeochemical cycling of key elements by coupling the reduction of insoluble metal oxides to the oxidation of the organic carbon. Microbial metal reduction has been identified as an effective means for immobilizing heavy metals and radionuclides in situ thus preventing their migration in the environment. Among metal ion reducing bacteria, *Shewanella oneidensis* MR-1 is notable due to its extensive respiratory versatility. In addition to O_2, this bacterium can respire various organic and inorganic substrates, including fumarate, nitrate, nitrite, thiosulfate, elemental sulfur, trimethylamine *N*-oxide (TMAO), dimethyl sulfoxide (DMSO), anthraquinone-2,6-disulphonate (AQDS), as well as various soluble and solid metal electron acceptors such as chromium, cobalt, iron, manganese, technetium, uranium, and vanadium (12, 27, 34).

Analysis of the *S. oneidensis* MR-1 genome sequence predicts a branched electron transport system that contains 42 putative *c*-type cytochromes and supports the proposed complexity of the energy-generating pathways in this organism (20, 30) Gene expression and environmental sensing in this organism is governed by 88 two-component regulatory and 27 methyl-accepting chemotaxis proteins suggesting a relatively robust system for detecting and responding to changes in extracellular conditions. A diverse transport network (20) including various peptide uptake systems, amino acid efflux pumps, and transporters for a wide range of metal cations has also been putatively identified. Notably, there are nine predicted multidrug efflux proteins from the resistance-nodulation-cell division (RND) family in MR-1, more than in any microorganism investigated to-date, other than *Pseudomonas aeruginosa* (20, 46). Although the precise cellular role of these multidrug transporters is yet to be determined, previous results indicate that a number of *S. oneidensis* MR-1 genes involved in drug resistance display increased mRNA levels under Fe(III)-reducing conditions (6).

In this study, we compared mRNA expression patterns of *S. oneidensis* MR-1 in batch cultures exposed to different metal and nonmetal electron acceptors using whole-genome DNA microarrays. The results reported herein provide insights into the complex structure of the MR-1 respiratory chain and identify major pathway components implicated in anaerobic respiration. This work represents an important step towards understanding the anaerobic respiratory system of *S. oneidensis* MR-1 on a genomic scale and has produced numerous candidate genes for more detailed functional analyses.

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MATERIALS AND METHODS

Growth conditions. *S. oneidensis* MR-1 was routinely cultured at 30°C on Luria-Bertani broth (LB; pH 7.4) (43) or on modified basal minimal medium (pH 7.5) (33), containing 0.05 g/liter tryptone and 0.025 g/liter yeast extract. To increase the buffer capacity of the medium and prevent drastic changes in pH during anaerobic respiration, 30 mM HEPES was added to the medium. For microarray expression profiling experiments, 50-ml cultures of *S. oneidensis* MR-1 were grown aerobically on LB to mid-exponential phase (optical density at 600 nm [OD_{600}] of 0.8), pelleted by centrifugation (5,000 × *g* for 10 min) and resuspended in 900 ml of anaerobic minimal medium containing 15 mM sodium fumarate and 20 mM sodium lactate. The anaerobic fumarate-reducing cultures were grown overnight to early stationary phase (OD_{600} of 0.14 to 0.15), pelleted by centrifugation under anaerobic conditions, and concentrated threefold in minimal medium containing 20 mM sodium lactate. The cell suspensions were then divided into 20-ml aliquots and incubated at 30°C for 3.5 h under 100% N_2 atmosphere in the presence of a single electron acceptor. The electron acceptors used included ferric citrate (10 mM), ferric oxide in the form of hydrous ferric oxide (HFO) (10 mM), manganese dioxide (10 mM), colloidal manganese(IV) (10 mM), cobalt(III)-EDTA (200 μM), dimethyl sulfoxide (DMSO; 10 mM), trimethylamine *N*-oxide (TMAO; 10 mM), disodium thiosulfate (10 mM), sodium nitrate (5 mM), and fumarate (10 mM) as a reference. The colloidal and oxide forms of Mn(IV) were prepared as described previously (11, 36). Cultures exposed to ferric citrate, HFO, manganese dioxide, colloidal manganese, and Co(III)-EDTA are hereafter collectively referred to as metal-reducing conditions, while cultures incubated in the presence of O_2, thiosulfate, nitrate, TMAO, and DMSO are referred to as non-metal reducing. For comparison purposes, a set of 20-ml shake-flask cultures supplemented with 20 mM sodium lactate were incubated aerobically for 3.5 h at 30°C with 180 rpm shaking. Although further in the text we refer to them as aerobic, we acknowledge that they were, in all likelihood, O_2-limited cultures. This limitation commonly exists in batch cultures for which mass transfer kinetics from gas to liquid phase does not exceed rapid specific O_2 consumption rates associated with bacterial respiration during mid-exponential phase.

Global expression analysis. Microarray expression profiling was carried out using *S. oneidensis* MR-1 whole-genome microarrays which contained a total of 4648 elements corresponding to unique segments of individual open reading frames (ORFs) (19). All microarray procedures including total RNA isolation, cDNA labeling and microarray hybridization were carried out using previously published protocols (5). The arrays were visualized by ScanArray ExpressHT confocal laser scanner (PerkinElmer, Boston, MA), and hybridization signal intensities were quantified using ImaGene software (Biodiscovery, El Segundo, CA). Statistical analyses and data mining were carried out using ArrayStat v.2 (Imaging Research, St. Catharines, ON) and GeneSpring v.7.0 (Silicon Genetics, Redwood City, CA).

Three biological replicates obtained from independently treated *S. oneidensis* MR-1 cultures were used in the gene expression analysis for all growth conditions. In addition, each microarray slide contained duplicate sets of gene fragments, and the RNA isolated from each replicate sample was hybridized with two microarrays using fluorescent-dye reversal. This resulted in a total of 12 data points for each condition enabling the use of rigorous statistical tests to determine significant changes in gene expression. Expression level-based filtering was carried out to eliminate genes with fluorescent signal intensity lower than two standard deviations above the background (39). Within the proportional model selected for statistical testing, the pooled common error method was used to remove data that were poorly reproducible, yet had a disproportionately large effect on statistical results. Non-linear intensity dependent normalization was used to standardize the microarray data and eliminate any dye-related and intensity-dependent artifacts (51). To remove genes that did not vary significantly across different respiratory conditions, one-way analysis of variance and false discovery rate testing correction (7) were performed. Additional validation of the microarray data was performed using quantitative real-time PCR (qPCR) which was carried out as described previously (19). The linear correlation between quantitative PCR and microarray data was performed based on the linear mean values using SigmaPlot 8.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Experimental design and validation of the microarray data. Global transcriptome profiles displayed by *S. oneidensis* MR-1 cultures subjected to different electron acceptors were determined using DNA microarrays representing ~99% of the total protein-coding capacity of the MR-1 genome (19). Relative expression ratios were derived by comparing mRNA abundance levels in cells induced by O_2, nitrate, thiosulfate, DMSO, TMAO, ferric citrate, HFO, manganese dioxide, colloidal manganese, and cobalt relative to mRNA levels in fumarate-exposed cells. Fumarate-respiring conditions were selected for the baseline to facilitate the identification of genes responding to specific anaerobic electron acceptors as opposed to a more general aerobic to anaerobic transition. Since the reduction kinetics, energy yields and specific growth rates can vary for different electron acceptors, cultures were harvested after 3.5-hr incubations to distinguish between the putative electron acceptor-induced changes in gene expression and growth-related effects.

To validate the microarray data, qPCR was used to evaluate 10 *S. oneidensis* genes that exhibited differential expression and were predicted to be encoded within one of four different operons. Results obtained for all 10 genes were in accordance with results obtained by microarray hybridization with average Pearson correlation coefficient $r = 0.92 \pm 0.11$ (see Table S1 in the supplemental material). Moreover, both qPCR and microarray data demonstrated a significant correlation ($r = 0.90$) in expression patterns among genes putatively localized within the same operon.

General patterns of expression in response to different electron acceptors. Among genes displaying statistically different expression levels, we identified a total of 2827 genes showing ≥2-fold change in relative mRNA abundances in at least one of the electron acceptor-exposed cultures, relative to fumarate (Table S2 in the supplemental material). In general, the number of differentially regulated genes did not vary significantly across different electron acceptors with the exception of TMAO- and DMSO-reducing cultures, where the latter accounted for only 81 genes (Fig. 1A). While this observation likely reflects fundamental differences in the pathways involved in sensing and utilization of organic and inorganic electron acceptors, it could have also resulted from using an organic electron acceptor (i.e., fumarate) as a reference condition for all comparisons.

Similar distribution patterns were observed for the majority of functional categories; however, there were several exceptions, most notably among genes encoding amino acid biosynthesis, protein synthesis, and energy metabolism functions (Fig. 1B, C, D; see also Tables S3, S4, and S5 in the supplemental material). In particular, genes predicted to encode functions affiliated with the biosynthesis of glutamate (*arg*, *gln*, *glt*) and pyruvate (*ilv*, *leu*) family amino acids displayed 2- to 20-fold downregulation under metal-reducing conditions while exhibiting substantial activation in the presence of nitrate. At the same time, the mRNA levels of genes encoding putative ribosomal proteins (*rpl*, *rpm*, *rps*) were significantly increased in the presence of non-metal electron acceptors, specifically O_2 and thiosulfate. The non-metal electron acceptors also positively affected the expression of genes involved in energy metabolism, while the majority of the genes in this functional category were downregulated in the presence of metals. In particular, specific activation of genes encoding putative *c*-type cytochromes (*cco*, *cyo*) and other electron transfer-related proteins (*hyd*, *psr*, *fdn*) was observed under thiosulfate- and nitrate-reducing conditions. Within other role categories, pre-

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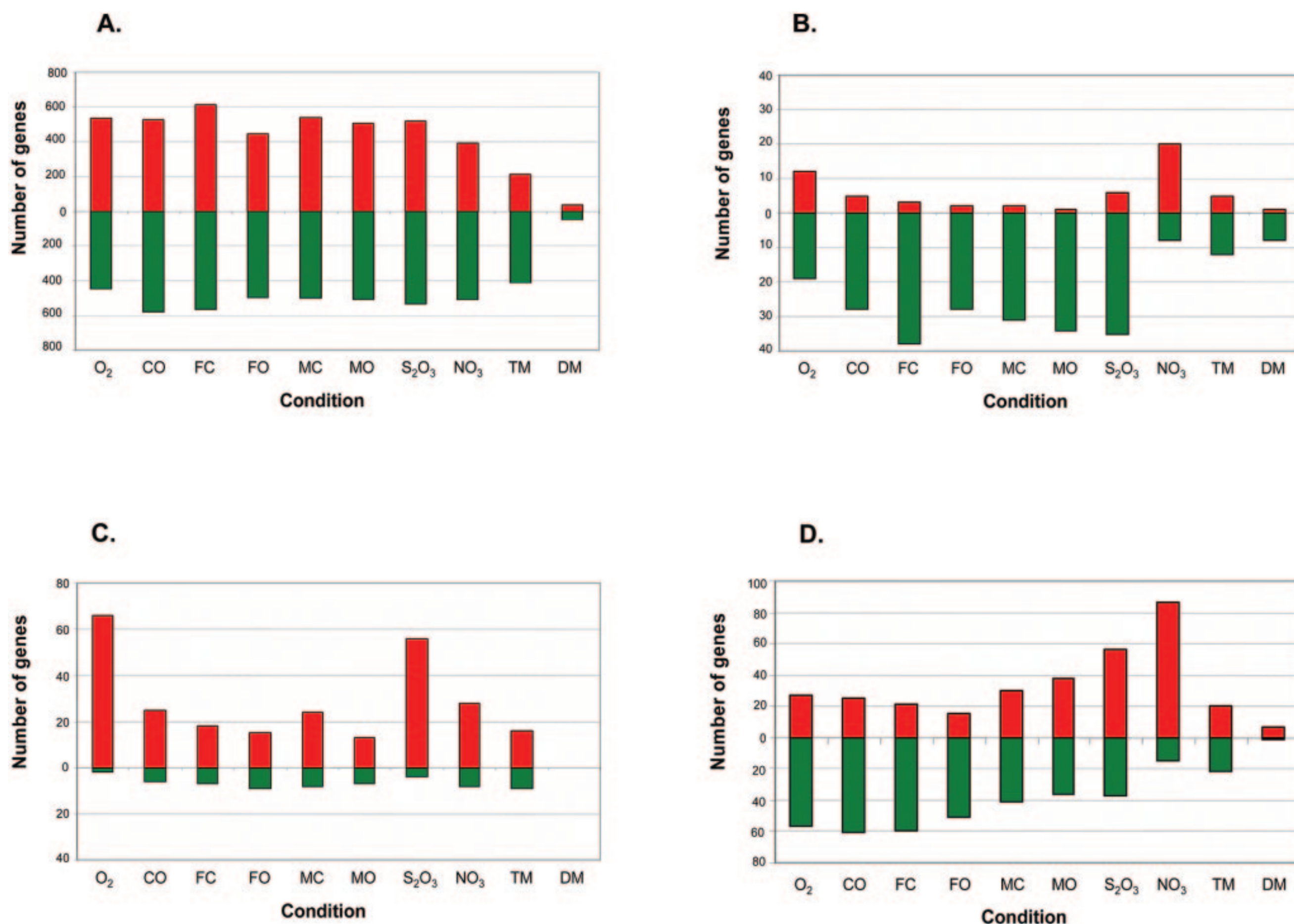

FIG. 1. Distribution of 2827 differentially expressed *S. oneidensis* MR-1 genes across different electron acceptor conditions (A). Distribution of *S. oneidensis* MR-1 genes involved in amino acid biosynthesis (B), protein synthesis (C), and energy metabolism (D) across different electron acceptor conditions. The functional classification corresponds to the main role categories assigned in the TIGR database (http://www.tigr.org/tigr-scripts/CMR2/gene_table.spl?db = gsp). Electron acceptor abbreviations: O_2, oxygen; CO, Co(III) EDTA; FC, ferric citrate; FO, hydrous ferric oxide; MC, colloidal manganese(IV); MO - manganese dioxide; S_2O_3, sodium thiosulfate; NO_3, sodium nitrate; TM, trimethylamine *N*-oxide; DMSO, dimethyl sulfoxide. Red bars indicate up-regulated genes, green bars represent down-regulated genes.

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dicted regulatory genes showed preferential upregulation, while the majority of genes encoding signal transduction and sensory proteins were downregulated under metal-reducing conditions.

To identify the dominant expression traits displayed by *S. oneidensis* MR-1 under various respiratory conditions, we used singular value decomposition technique (known also as principal component analysis) to produce a set of patterns, or "eigengenes", linear combinations of which can be assembled to represent the behavior of the MR-1 transcriptome (1). Interestingly, the most significant components associated with the observed genome-wide effects were represented by "eigengenes" activated or repressed in the presence of metals that captured 14.6% and 55.8%, respectively, of the overall expression variation in the experiment. Furthermore, striking similarities were observed between these two top components and the expression profiles of key regulatory genes including six that encode putative sigma factors (Fig. 2). The latter included *rpoS* (σ^{38}) (SO3432) and *rpoN* (σ^{54}) (SO3961) genes which showed significant downregulation in the presence of metals and thiosulfate, while *rpoH* (σ^{32}) (SO4583) and *rpoE* (σ^{24}) (SO1342) were induced under the same conditions. In addition to these well-known regulators, two other genes encoding putative sigma factors (SO1986 and SO3096) displayed a 4- to 12-fold increase in mRNA levels under metal-reducing conditions. Sequence analysis indicated that both genes belong to the σ^{70} extracytoplasmic function (ECF) family of sigma factors that function in a manner similar to two-component systems by coordinating cytoplasmic transcriptional responses to signals perceived by protein domains external to the cytoplasmic membrane (23). As a class, ECF sigma factors play key roles in coordinating gene transcription during various stress responses and morphological development (21, 23) and, as shown recently, in regulation of different iron acquisition systems (50).

Metal-induced shifts in transcriptome patterns of *S. oneidensis* MR-1. The extent of the MR-1 transcriptome response to metal ions was further revealed by hierarchical clustering analysis. A high degree of similarity in global expression profiles was exhibited throughout all metal-reducing conditions,

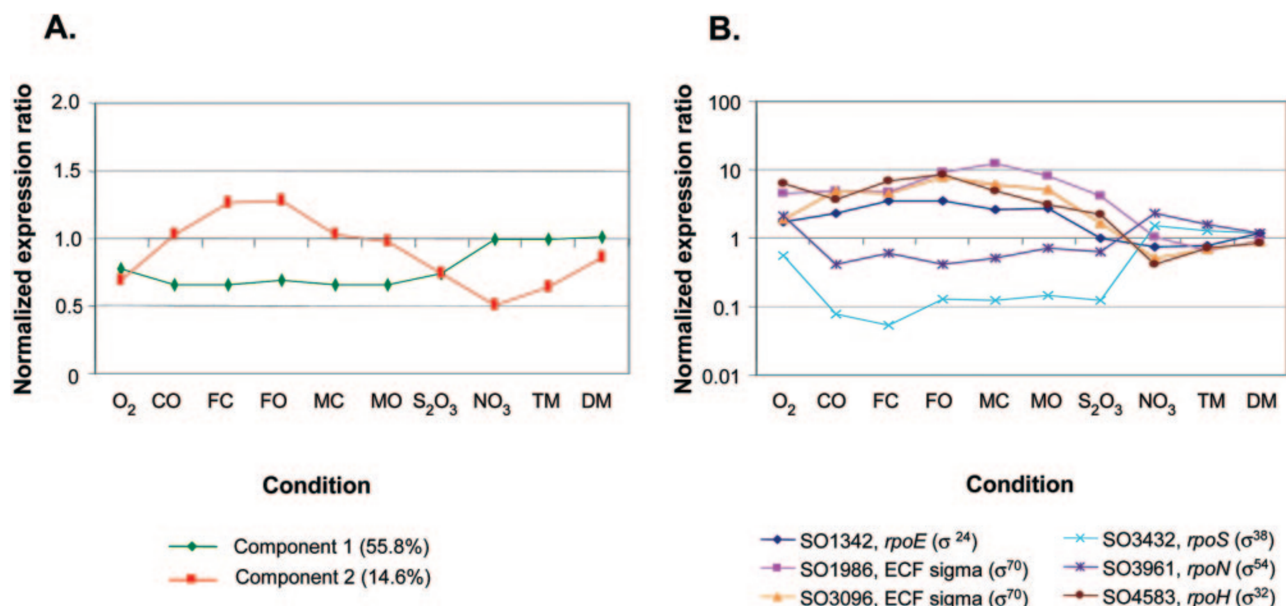

FIG. 2. Correlation between the top 2 principal components representing the behavior of the *S. oneidensis* MR-1 transcriptome (A) and expression profiles of 6 *S. oneidensis* genes encoding putative sigma factors (B). Relative transcript abundances represent a ratio of mRNA levels in test cultures to these displayed by the fumarate-grown reference. Values of >1 indicate mRNA abundance increase under test conditions; values of <1 indicate decrease under test conditions. Electron acceptor abbreviations: O_2, oxygen; CO, Co(III) EDTA; FC, ferric citrate; FO, hydrous ferric oxide; MC, colloidal manganese(IV); MO, manganese dioxide; S_2O_3, sodium thiosulfate; NO_3, sodium nitrate; TM, trimethylamine *N*-oxide; DMSO, dimethyl sulfoxide.

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resulting in profiles that grouped separately from non-metal electron acceptors and formed a tight, well-defined branch (Fig. 3). Remarkably, no significant differences in gene expression profiles were found between solid (HFO, MnO_2, colloidal Mn) and complexed soluble (ferric citrate, Co(III)-EDTA) metal acceptors. In accordance with the PCA results (Fig. 2), we identified two major expression groups that displayed activation and repression in the presence of metals and accounted for over 60% of the differentially expressed genes (clusters I and V, respectively) (see also Tables S6 and S7 in the supplemental material). While genes encoding hypothetical and conserved hypothetical proteins dominated both clusters, there were several functional subgroups that were characterized by their specific response to metal electron acceptors. In cluster V, a significant decrease in expression levels was displayed by the putative 42-kb flagella biosynthesis cluster (*flg*, *flh*, *fli*), amino acid biosynthesis genes (*arg*, *gln*, *glt*, *ilv*, *leu*), as well as various components of the electron transport chain such as the NADH dehydrogenase (*nuo*), Na-translocating NADH:ubiquinone oxidoreductase (*nqr*), and cytochrome *c*- and cbb_3- oxidases (*cyo*, *cco*). The *mtrCAB* operon which encodes two decaheme *c*-type cytochromes and an outer membrane protein linked to Fe(III) and Mn(IV) respiration in *S. oneidensis* MR-1 (3, 4) also showed 2- to 8- fold decrease in mRNA levels under metal-reducing conditions.

Approximately 40% of genes specifically induced in response to metal respiratory conditions in *S. oneidensis* MR-1 encoded hypothetical or conserved hypothetical proteins, while others encoded putative proteins involved in energy metabolism, detoxification, toxin resistance and regulatory functions. SO3300, predicted to encode a split flavocytochrome *c*, was the only *c*-type cytochrome of *S. oneidensis* MR-1 that uniquely displayed significantly elevated transcript levels across all metal-reducing conditions. Although homologs of SO3300 were not found in the recently sequenced genomes of several *Shewanella* strains (for more information see http://www.jgi.doe.gov/sequencing/DOEmicrobes.html) other fused flavocytochrome *c* proteins have been implicated in metal reduction. In particular, a fused flavocytochrome *c* (IfcA), was demonstrated to be induced by iron and repressed by fumarate and oxygen in *S. frigidimarina* (41). The closest homolog of IfcA in *S. oneidensis* MR-1 is encoded by SO1421. However, SO1421 showed only ≤2-fold induction under metal reducing growth conditions. The increased expression of SO3300 compared to SO1421 under metal reducing conditions suggests that the former may play either direct or indirect role in metal reduction.

Among genes involved in detoxification and drug/toxin resistance, putative operons encoding arsenical resistance system (SO0532 to SO0534) and MexF-MexE efflux pump proteins (SO3492 and SO3493) exhibited specific upregulation in the presence of metal electron acceptors. The former, which included the putative arsenate reductase, the arsenical efflux pump and the arsenical operon repressor, is homologous to the *Escherichia coli ars* detoxification system (32) and, as shown recently, is advantageous for respiratory As(V) reduction by *Shewanella* strain ANA-3 by protecting the organism from high heavy-metal concentrations (42). In contrast, the physiological function of *mexFE* genes, which on average displayed 4- to 40-fold induction under metal-reducing conditions, is largely unknown in *Shewanella*. The predicted products of *mexFE* belong to the RND-family of efflux transporters, which as a group are proposed to catalyze substrate efflux across the inner membrane to the cell surface via a proton antiport mechanism.

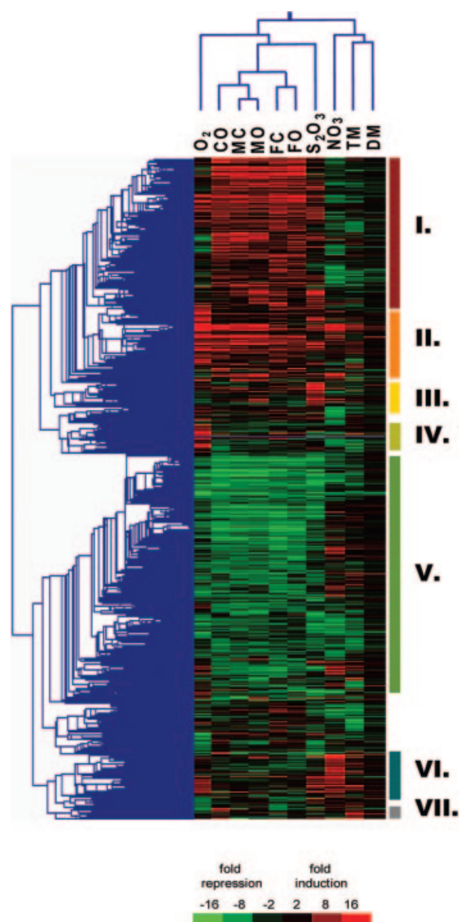

FIG. 3. Hierarchical clustering of 2827 *S. oneidensis* genes exhibiting differential expression in cultures exposed to metal and non-metal electron acceptors. Each column indicates individual electron acceptor. Relative transcript abundances represent a ratio of mRNA levels in test cultures to these displayed by the fumarate-grown reference. Electron acceptor abbreviations: O_2, oxygen; CO, Co(III) EDTA; FC, ferric citrate; FO, hydrous ferric oxide; MC, colloidal manganese(IV); MO, manganese dioxide; S_2O_3, sodium thiosulfate; NO_3, sodium nitrate; TM, trimethylamine *N*-oxide; DMSO, dimethyl sulfoxide.

While the substrate specificity of this particular transporter system is unknown, homologous efflux pumps, including the *S. oneidensis* MR-1 TolC system (44), have been described in several gram-negative organisms where they play a role in export of and resistance to multiple antimicrobial agents, heavy metals, and organic solvents (37). In particular, the observation that TolC-dependent efflux system of MR-1 is required for protection against toxic levels of AQDS and other redox active compounds may provide direct evidence linking efflux pumps with the reduction of insoluble electron acceptors (44).

As suggested by the global mRNA patterns, upregulation of a large number of putative regulatory genes was observed in metal ion-supplemented cultures. While no functional assignments have been made within this group, high induction levels were observed for genes encoding putative proteins that belong to GntR- (SO2282), HlyU-(SO3538), LuxR- (SO3305), and TetR- (SO1393, SO3743, SO4326, and SO4468) family of regulators. Among the few sensory and chemotaxis genes of *S. oneidensis* MR-1 that displayed specific upregulation under metal-reducing conditions, three putative PAS domain-containing signal transducers were identified (SO0141, SO0544, and SO1385, (48). One of them, SO1385, displayed significant similarity (39% identity, 58% homology over 502 amino acids) to an *E. coli* Aer aerotaxis transducer which is an example of a well characterized PAS domain-containing sensory protein which monitors the respiratory status of the cell via redox changes in a component of the electron transport system (8). Moreover, the conservation of amino acid residues in the putative PAS domain of SO1385 that are thought to be critical for FAD binding and aerotaxis (40) strongly suggests that this protein is, in fact, an FAD-containing redox/energy taxis transducer (47). The presence of such a sensory mechanism would seem particularly beneficial in *S. oneidensis* MR-1 for communicating sensory adaptation during growth and respiration of mineral forms of Fe(III) and Mn(IV) which are poorly soluble under oxic, circumneutral pH conditions.

It is noteworthy that the vast majority of genes upregulated in the presence of metals displayed decreased mRNA levels under nitrate-reducing conditions. The only group that showed upregulation under both nitrate- and metal-reducing conditions fell into a 287-gene cluster (cluster II) (see also Table S8 in the supplemental material) that contained a large number of stress-response related proteins. Among these, significant induction levels were displayed by genes putatively regulated by RpoH (σ^{32}), an alternative sigma factor that has been confirmed to play a central role in the heat shock response of *S. oneidensis* MR-1 (19). Out of 30 genes bearing the putative σ^{32} consensus sequence and upregulated by heat shock stress, increased expression levels under nitrate- and metal-reducing conditions was displayed by those encoding putative molecular chaperones (SO0703, SO0704, SO1126, and SO1127), ATP-dependent proteases (SO3577, SO4162, and SO4163), thioredoxins (SO0406, SO0452, and SO2017), and an alkyl hydroperoxide reductase (SO0958) (19). Oxidative stress studies in other bacterial species showed that the latter possesses a redoxin activity (13) and plays an important role in protection against superoxide radicals produced via organic peroxides (18, 22). Our results indicate that the putative alkyl hydroperoxide reductase (*ahpC*) of *S. oneidensis* MR-1 exhibits 10- to 50-fold increase in mRNA abundances under Co(III)-, Mn(VI)-, thiosulfate- and nitrate-reducing conditions. Since these expression levels are significantly higher than those shown by any other radical-scavenging system in MR-1, it is likely that AhpC contributes to the removal of toxic radicals generated during anaerobic respiration.

Specific activation of non-metal electron acceptor respiratory systems. Although the vast majority of genes within the 2827-gene data set displayed significant responses to metals, hierarchical clustering analysis identified groups specifically upregulated under thiosulfate, aerobic, nitrate, and TMAO-reducing conditions that corresponded to clusters III, IV, VI, and VII, respectively (Fig. 3; see also Tables S9, S10, S11, and S12 in the supplemental material). Among those, nitrate produced the strongest transcriptome response resulting in specific upregulation of over 160 *S. oneidensis* MR-1 genes. Significant induction levels were displayed by the putative nitrate-inducible Se-containing formate dehydrogenase (SO0101–0105), peri-

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plasmic nitrate reductase (SO0845–0849), and the periplasmic nitrite reductase (SO3980) genes, whose role in anaerobic respiration has been well defined in other bacteria (16, 17, 45). Expression data also indicated upregulation of several amino acid and cofactor biosynthesis operons including the one involved in molybdenum transport and cofactor biosynthesis (SO4447 to SO4450). In addition, genes coding for enzymes of sulfur metabolism, *i.e*., cysteine desulfurase (SO2264 and 2265), sulfate transport (SO3599-3561), and assimilatory sulfate reduction (SO3723 to 3727, SO3736 and 3737) showed specific activation in the presence of nitrate. While such responses were expected given the requirement for molybdenum and Fe-S cofactors in the activity of nitrate and nitrite reductases, respectively, the elevated mRNA levels of genes encoding TCA cycle enzymes (SO0343, SO1930 to 1933, SO2629) cannot be readily explained. It seems likely however, that simultaneous upregulation of genes encoding Na-translocating NADH:ubiquinone oxidoreductase (SO0904 to 0907) and NADH dehydrogenase (SO1013 to 1020) reflects the activation of the TCA cycle and, specifically, the increase in NAD^+ reduction by isocitrate and 2-oxoglutarate dehydrogenases. Furthermore, the upregulation of detoxification genes, in particular, cytochrome c_{551} peroxidase (SO2178) and catalase/peroxidase HPI (SO4405), may contribute to the removal of toxic free radicals generated by the TCA cycle and the nitrate respiration intermediates.

While the dissimilatory reduction of sulfur-containing compounds by *S. oneidensis* MR-1 is well-documented (31, 34), the mechanism and mediators of this process in MR-1 are still to be determined. Studies in other organisms have revealed that reduction of inorganic sulfur species occurs through reductive cleavage of the sulfur-sulfur bonds catalyzed by molybdopterin guanine dinucleotide (MGD) cofactor-containing enzymes such as tetrathionate (Ttr), thiosulfate (Phs) and polysulfide (Psr) reductases (21). Our data indicate that genes encoding at least one of these enzymes, *psrABC* (SO4060–4062), displayed 8- to 16-fold preferential upregulation under thiosulfate-reducing conditions. Among other *S. oneidensis* MR-1 genes exhibiting $S_2O_3^{2-}$-specific increase in mRNA levels, we identified a 4-gene cluster containing three putative mono-heme *c*-type cytochromes and a predicted molybdopterin-binding oxidoreductase (SO0714–0717) homologous to putative sulfite oxidoreductases from *Ralstonia metallidurans* CH34, *Chromobacterium violaceum*, and *Thermus thermophilus* HB27. A specific 3- to 30-fold induction was observed for a 12-gene cluster (SO0476 to 0488) encoding a predicted hepta-heme cytochrome *c*, three specialized cytochrome *c* biogenesis proteins, and a membrane bound NrfCD-like electron transfer complex associated with four putative copper transport/processing proteins (29). Other *c*-type cytochromes strongly induced by thiosulfate included the split-soret diheme *c* (SO0939), the monoheme *c* (SO4142), and the hypothetical triheme *c* (SO4572), bringing the total number of putative *c*-type cytochromes induced by thiosulfate to nine, the most induced by any treatment used in our study. Finally, homologs for *hydAB* and *fdh* genes (SO3920–3922), which encode a putative [Fe] hydrogenase and formate dehydrogenase, respectively, displayed four- to sixfold upregulation under thiosulfate-reducing conditons.

A relatively weaker transcriptome response was displayed by *S. oneidensis* MR-1 cells incubated with TMAO. With the exception of *torDAC* (SO1231–1233) and *torF* (SO4694) genes which encode components of the trimethylamine *N*-oxide reductase and a hypothetical protein, respectively, the majority of genes upregulated in the presence of this electron acceptor displayed only a marginal response. Our microarray data complement the results of recent studies which identified the TorS/TorR two-component system as the key component involved in TMAO-dependent regulation in *S. oneidensis* MR-1 (9). In these studies real-time PCR and genetic footprinting analyses revealed that TorR recognizes direct repeat nucleotide sequence TTCATAN$_4$TTCATA found in the promoters of *torCAD*, *torF*, and *torR*, that allow TMAO-dependent induction of the *torCAD* and *torF* units and repression of the *torR* gene (9).

Neither of the genes predicted to encode putative dimethyl sulfoxide reductases, *dmsAB*-1 (SO1429, SO1430) and *dmsAB*-2 (SO4358, SO4357), were significantly induced by DMSO. While the former displayed 3- to 8-fold upregulation under thiosulfate-reducing conditions, the relative mRNA abundance levels of the latter remained unchanged across all respiratory conditions. Analysis of the deduced amino acid sequences of *dmsA*-2 revealed a presence of mature N terminus (CSGCKTC) characteristic of outer membrane lipoproteins, suggesting this gene does not encode the classic DMSO reductase which is normally localized in the inner membrane. In contrast, deletion of *S. oneidensis dmsA*-1 results in severe growth deficiency and loss of DMSO reductase activity in MR-1 (D. Saffarini, unpublished results). While our results suggest that *dmsAB*-1 operon is involved in DMSO reduction, its upregulation under thiosulfate-reducing conditions may be indicative of a broad substrate specificity of the DMSO reductase complex in MR-1.

Consistent with our previous notion that shake-flask aerobic cultures were, in fact, suboxic, O_2-exposed cells of *S. oneidensis* MR-1 did not display any activation of genes specific for aerobic respiration. Specific upregulation, however, was observed for genes involved in Sec-dependent protein translocation (SO1192–01196, SO3110–3113), cytochrome *c* maturation (SO0259, SO0260, SO0264), and proline biosynthesis (SO3354–3357). Among transcriptional regulators, O_2-dependent induction was displayed by genes encoding putative *spo*IIA family (SO3950) and aerobic respiration control (*arcA*, SO3988) proteins. In addition, over fourfold increase in relative mRNA levels was observed for the putative ferritin gene (SO0139). While being ideal Fe(III) storage proteins, ferritins can also decrease iron toxicity and reactive oxygen species (46) production generated via the Fenton reaction (24). In *S. oneidensis* MR-1, where high iron demand results in increased intracellular iron concentrations (15), the reactivity of Fe(III) may be counteracted by sequestering the metal into ferritins, which can harbor up to 4500 iron atoms per molecule (14).

Conclusions. To define the repertoire of genes specifically responding to different respiratory conditions in *S. oneidensis* MR-1, global mRNA patterns were examined in cells exposed to a range of terminal electron acceptors. Comparison of gene expression patterns induced under non-metal and metal-reducing conditions allowed us to identify a clear yet remarkably similar global gene expression response by MR-1 to the various metals provided as electron acceptors. While the response

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mechanisms undoubtedly involve reorganization of regulatory networks and metabolic pathways, the potential toxicity of the metal reduction products, i.e., divalent metal cations must be considered. The 3.5-hr exposure to different electron acceptors in this experiment was specifically selected to limit cell division and growth, however the metabolic activity of cells incubated in a closed system would have resulted in an accumulation of reduced metals (Mn^{2+}, Fe^{2+}, or Co^{2+}) in the medium. Our estimates, using data from previous kinetic studies with similar cell densities, suggest that at least 5 mM of the Fe(III) citrate and at least 100 μM HFO would have been reduced within the 3.5 h incubation period (27). Many bacteria have developed diverse and efficient mechanisms for dealing with the toxic effects of metal cations and we anticipate that MR-1 has such capabilities. This notion is consistent with the upregulation of putative RND protein family genes that have been implicated in cation export and can confer heavy metal resistance (35). It is also interesting to note that for genes encoding the arsenic detoxification system the relative expression ratios are generally higher for the soluble metal complexes, such as Co(III)-EDTA and ferric citrate, than for the solid phase oxides, a finding consistent with more rapid reduction of the complexed metals and accumulation of higher concentrations of reduced metals during the induction period. In addition to the RND family, the upregulation of *rpoH* and *rpoE* genes along with two other ECF sigma factors and stress-response related genes in the presence of metal electron acceptors is consistent with an accumulation of divalent metal cations that can trigger global stress-related response leading to induction of a variety of detoxification, resistance and transport functions (10). Such coordinated expression of stress response and detoxification mechanisms in *S. oneidensis* MR-1 can be a key factor in adapting to anoxic metal-reducing conditions in aquatic sediment and submerged soil systems where substantial amounts of Fe(II) and Mn(IV) can be generated (2, 26, 28).

Induction of stress-associated genes observed in response to metals correlated with a high sensitivity of *S. oneidensis* MR-1 to both low-dose ionizing radiation and nitrite ((15, 38); Klappenbach, unpublished data). Of particular interest was the nitrate-dependent upregulation of several genes involved in sulfur acquisition that were not induced in the presence of thiosulfate. The activation of sulfur metabolism, concomitant with the induction of genes involved in the stress response during nitrate reduction are a likely consequence of nitrite accumulation during dissimilatory reduction of nitrate to ammonia. *S. oneidensis* MR-1 reduces nitrate completely to nitrite prior to initiation of nitrite reduction to ammonia, and nitrite levels > 2 mM are toxic to cells during anaerobic growth (J. Klappenbach, unpublished data). Nitrite is a strong oxidant and elicits a stress response similar to ROS in *E. coli* (25). Upregulation of sulfur metabolism genes likely reflects a strong cellular demand for sulfur necessary for the synthesis of iron-sulfur centers irreversibly damaged by nitrite. Furthermore, activation of amino acid biosynthesis and TCA cycle genes in *S. oneidensis* MR-1 during nitrate reduction is congruent with observations of ROS-induced stress in *E. coli* that specifically affects dehydratases involved in branched-change amino acid biosynthesis and the TCA cycle (24).

The respiratory versatility of *S. oneidensis* MR-1, apparently conferred by a complex network of redox proteins including cytochromes, appears to be accompanied by a physiological trade-off in susceptibility to oxidative damage. In the environment, *Shewanella* spp. are most-often found in chemical gradients of electron acceptors such as metals, nitrate, and oxygen (49). Achieving a balance in environmental gradients between thermodynamic optima and minimization of oxidative damage is ostensibly one of the keys to the ecological success of *Shewanella*. Although the batch-culture approach did not allow us to unambiguously differentiate changes in expression between energy-generating pathways and toxicity-related effects for specific metals in the current study, the findings from this research will allow the design of experiments positioning *S. oneidensis* MR-1 in a physiological state maximizing energetic demands while minimizing oxidative damage. Future work will focus on comparisons of continuous steady-state cultures where cell growth and physiological state are more precisely controlled and characterized. Given the complexity of the electron transport system of this organism, the results from this research illustrate that coordinate regulation will be the building blocks on which we begin to understand the complex regulation of energy-generating processes and, in fact, will allow us to predict other genes that may behave in similar ways through genomic analysis.

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