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# The Response of *Desulfotomaculum reducens* MI-1 to U(VI) Exposure: A Transcriptomic Study

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***Desulfotomaculum reducens*** **is the first Gram-positive sulfate- and metal- reducing bacterium for which the transcriptomic response to uranium exposure has been evaluated. The genes upregulated during fermentative growth in the presence of U(VI) as compared to its absence included those encoding for proteins involved in respiration such as NADH quinone oxidoreductase and heterodisulfide reductase. This finding suggested that electrons were shuttled to the electron transport chain during fermentation and points to the reduction of U(VI) as a metabolic process. Although U(IV) is typically insoluble and readily removable by filtration, U(IV) produced during active growth was not retained by a 0.2 $\mu$m pore size filter and filtration was not sufficient to differentiate between U(VI) and U(IV). In addition, genes involved in iron homeostasis were upregulated in the presence of uranium, which was consistent with the upregulation of genes involved in *c*-type cytochrome biogenesis. Despite the upregulation of cytochrome biosynthesis genes, the sole *c*-type cytochrome encoded in the genome was not differentially expressed. Finally, genes encoding metal efflux pumps were also upregulated indicating the toxic nature of uranium. Analysis of the time-dependent gene expression showed that sporulation was the dominant process at the early stationary phase and that the presence of U at that stage did not impact expression.**

**Keywords** uranium, *Desulfotomaculum reducens*, transcriptomics, metal reduction, spore-former

## INTRODUCTION

Uranium contamination is a widespread environmental problem around the world (Wall and Krumholz 2006). Stimulating indigenous microbial communities to carry out the *in situ* reduction of soluble U(VI) to insoluble U(IV) has been proposed as a potential bioremediation strategy for these contaminated sites (Anderson et al. 2003; Madden et al. 2009; N'Guessan et al. 2008; Wu et al. 2007; Yabusaki et al. 2007). However, effective bioremediation requires an understanding of the mechanisms involved in bacterial metal reduction and of the response of microorganisms to metal toxicity. Transcriptomics, the simultaneous analysis of gene expression of an entire genome in response to a specific condition, offers the opportunity to unravel the molecular mechanisms involved in both microbial metal reduction and metal toxicity responses in bacteria.

Several studies have carried out whole-genome transcriptional analyses of either heavy metal stress responses or biological metal reduction for model microorganisms. For example, in the case of *Shewanella oneidensis* MR-1, a facultative Gram-negative gammaproteobacterium, gene expression profiling during chromium and uranium reduction revealed the involvement of multiple *c*-type cytochromes (Bencheikh-Latmani et al. 2005). The findings were confirmed by the analysis of the phenotypes of appropriate mutants. The stress response to chromium and strontium was also studied in *S. oneidensis* (Brown et al. 2006a, 2006b).

The response of a mid-exponential phase culture of strain MR-1 to a toxic concentration of chromate (1 mM) over time was very complex. It appeared to involve the induction of genes with functions such as iron uptake, sulfur assimilation, DNA repair, as well as the downregulation of genes involved in energy metabolism (Brown et al. 2006b). Exposure of a mid-exponential phase culture to strontium resulted in the upregulation of genes primarily involved in siderophore-mediated iron sequestration and transport despite the presence of sufficient iron in the medium. This response was specific to Sr but it remains unclear whether it corresponded to a detoxification mechanism (i.e., through $Sr^{2+}$ complexation by siderophores) or the indirect consequence of a failure in the iron homeostasis sensor Fur (Brown et al. 2006a).

Transcriptional analysis of the stress response of *Caulobacter crescentus*, a Gram-negative alphaproteobacterium, to chromium, cadmium, selenium or uranium exposure demonstrated the upregulation of several redox active metal-resistance genes as well as stress-response proteins. However, those proteins were upregulated differently in the presence of the various metals (Hu et al. 2005). One of the three genes encoding a superoxide dismutase (*sodA*) was the only gene with a known function that was upregulated in all conditions, suggesting that all four metals induced oxidative stress.

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The specific response of *C. crescentus* to uranium exposure involved the extracellular precipitation of uranium in the form of the uranyl phosphate mineral, autunite. A candidate protein (CC1295) annotated as a possible phosphatase, which was 5.4 times upregulated in the presence of uranium, was proposed to be involved in the biological precipitation process (Hu et al. 2005). Very recently, a study reporting the transcriptome of *Geobacter uraniireducens* cells in uranium-containing sediments showed the expression of genes known to be involved in Fe and U reduction (*c*-type cytochromes) as well as genes dedicated to coping with N and P limitation (Holmes et al. 2009).

In contrast to the model organisms mentioned above, *Desulfotomaculum reducens* MI-1 is a sulfate-reducing, spore-forming Gram-positive bacterium. *D. reducens* was isolated from marine sediments heavily contaminated with chromium from Mare Island Naval Shipyard in California, USA. Despite being a sulfate-reducing bacterium (SRB), it is able to grow by coupling the oxidation of organic compounds to the reduction of Fe(III) to Fe(II) (Tebo and Obraztsova 1998). It was originally reported to grow using U(VI) as a terminal electron acceptor in the presence of butyrate (Tebo and Obraztsova 1998), but in experiments performed using pyruvate, a fermentative substrate, growth was not coupled to the use of U(VI) as a terminal electron acceptor (Junier et al. 2009). It was also found that spores of *D. reducens* are able to catalyze the reduction of U(VI) when $H_2$, a product of pyruvate fermentation, is provided as an electron donor in cell-free medium collected after cell growth (Junier et al. 2009).

Recently, the genome sequence of *D. reducens* was completed and manually annotated. One of the most striking results was the paucity of *c*-type cytochromes in the genome of *D. reducens* (Junier et al. 2010a), which contrasts heavily with the abundance of *c*-type cytochromes in the genomes of other metal-reducing microorganisms such as *S. oneidensis* (Heidelberg et al. 2002), *G. sulfurreducens* (Methe et al. 2003) and *G. metallireducens* (Aklujkar et al. 2009). These proteins are known to be involved in metal reduction in those bacteria (Bencheikh-Latmani et al. 2005; Shelobolina et al. 2007).

The availability of the *D. reducens* genome sequence has allowed the design of customized arrays to study the transcriptomic response of *D. reducens* to different growth conditions. In this study, we analyzed its transcriptomic response to U(VI) exposure, while growing fermentatively on pyruvate and compared it to fermentative growth without U(VI) as well as to sulfate reduction with the same substrate (pyruvate). Comparison of these three data sets (i.e., fermentation of pyruvate in the presence and absence of U(VI) and sulfate reduction with pyruvate as an electron donor) was intended to address the following questions: What expression level response does the presence of U(VI) trigger? What metabolic activity and biochemical pathways may be recruited in the response to U(VI)? What does this response reveal in terms of the mechanisms of reduction/detoxification of U(VI)?

We found that despite no obvious differences in the growth profiles of cultures grown fermentatively in the presence and absence of U(VI), active upregulation of genes involved in energy metabolism, metal detoxification, and iron metabolism appears at the transcriptional level. Some of the corresponding proteins – a trimeric hydrogenase, heterodisulfide reductases, electron transfer flavoproteins and genes involved in *c*-type cytochrome biogenesis- could be part of a mechanism for U(VI) reduction by vegetative cells.

## MATERIALS AND METHODS

### Media and Growth Conditions

*Desulfotomaculum reducens* strain MI-1 was kindly provided by Anna Obraztsova (University of Southern California, Los Angeles, CA). Standard anaerobic conditions were used throughout the study (Balch et al. 1979). Widdel low phosphate (WLP) medium, modified from Widdel and Bak (1992), was used for all the experiments. The constituents of the WLP medium were as follows (per liter): $NH_4Cl$, 0.25 g; $CaCl_2.2H_2O$, 0.1 g; $MgCl_2.6H_2O$, 0.5 g; NaCl, 5 g; KCl, 0.5 g; and $KH_2PO_4$, 0.03 g, vitamins and trace elements. The anoxic medium was dispensed in 100 mL volumes into 200 mL glass serum bottles and autoclaved. The following solutions were added from sterile anaerobic stocks (final concentration): yeast extract, 0.05%; $NaHCO_3$, 30 mM; 1,4-piperazinediethane sulfonic acid disodium salt monohydrate (PIPES), 20 mM; pyruvic acid, 20 mM (unless indicated otherwise). For experiments with U(VI) and sulfate, 100 $\mu$M U(VI) (as uranyl acetate) and 20 mM sulfate (as $Na_2SO_4$) were added, respectively. The final pH of the medium was $7.2 \pm 0.2$.

All cultures were grown in a 100% $N_2$ atmosphere. All chemical compounds were obtained from Sigma-Aldrich unless otherwise indicated. Growth in the cultures was monitored by $OD_{600}$ or protein content (when mentioned). The experiments were run in 4 biological replicates. A 10% inoculum from a spore preparation (Junier et al. 2009) was used for inoculation in the experiment in the presence or absence of U(VI). A fermentation culture was used as inoculum (10%) in the case of growth using sulfate as terminal electron acceptor.

### RNA Extraction

The cultures were sampled (16 mL) for RNA extraction at the time point targeted for the expression analysis. Samples were collected in RNAse-free 50 mL Falcon tubes by centrifugation at 7000 $\times$ *g* for 7 min and resuspended in 400 $\mu$L of 3 mg/ml lysozyme in TE buffer (pH 8.0) and mixed by vortexing. After digestion for 10 min at room temperature 1.4 mL of Buffer RLT (Qiagen) containing freshly added 0.01% vol/vol $\beta$-mercaptoethanol, was added to the sample and mixed vigorously by vortexing. The homogenized cell lysates were stored at –80°C. After all the samples were collected, the cell lysates were thawed for 15 min at 37°C in a water bath to dissolve salts.

The samples were separated into four equal aliquots of 450 $\mu$L. RNA extraction was carried out using the RNeasy extraction kit (Qiagen) with on column DNAse treatment (Qiagen).

The quantity and quality of the RNA were evaluated with a Nanodrop spectrophotometer (Thermo Scientific). RNA from the four aliquots was combined and precipitated using 0.1 volume of 1 M sodium acetate and 2.5 volumes cold 95% ethanol, incubating overnight at –20°C. RNA was collected by centrifugation for 15 min at 12,000 × g at 4°C and washed with 75% ethanol. RNA was dried at 37°C for 20 min and resuspended in 30 $\mu$L RNAse free water. RNA concentration and quality were re-measured using the Nanodrop and the Bioanalyzer (Agilent). A total of 20 $\mu$g of total RNA per sample was sent to Nimblegen Systems (Roche) for cDNA synthesis, labeling with Cy3, and hybridization onto a custom-designed 4 × 77 K microarrays.

### Array Design

The oligonucleotide microarray used in this study was a customized 4 × 77 K microarray designed by Nimblegen system (Roche Inc.). The arrays were designed using the genomic sequence of *D. reducens* MI-1 available on the website of the Joint Genome Institute (http://genome.jgi-psf.org/desre/desre.home.html). The sequence was submitted to Nimblegen Systems Inc. for microarray design and manufacture using maskless, digital micromirror technology. Two replicates of the genome were included on each chip. An average of 10 different 60-base oligonucleotides (60-mer probes) representing 3,258 open reading frames (ORF) in the genome for a coverage of 98% (the finished genome is annotated to have 3,324 ORFs). A complete record of all oligonucleotide sequences used and raw and statistically treated data files is available in the NCBI Gene Expression Omnibus database under accession number GSE 22944.

### Microarray Hybridization, Data Analysis and Identification of Differentially Expressed Genes

NimbleGen Systems, Inc. performed array hybridization using their Hybriwheel technology. The arrays were analyzed using an Axon GenePix 4000B scanner with associated software (Molecular Devices Corp., Sunnyvale, CA).

Normalized RMA (Robust Multi Array analysis) signals were used for the analysis using with the software GeneSpring GX v7.3. Only "per gene" normalization was applied when the data were loaded into a customized one-color experiment in the analysis software. Biological replicates were evaluated by using a condition tree and PCA (Principal Components Analysis). In most cases, biological replicates showed high similarity (data not shown) and were combined by computing average expression values. However, two biological replicates (one sample from *uranium* T1 and one from *fermentation* T2) that did not group with the respective replicates were removed from the analysis. Also, for the samples taken at 44 h (T3) only one biological replicate from *fermentation* and three biological replicates from *uranium* could be obtained.

Quality control of the data was performed using the option "filtering on control signal" in GeneSpring. To achieve this filtering, the values obtained from the cross-error model were used as cutoffs to eliminate genes with no expression. Differentially expressed genes were obtained by using the "filtering on fold" tool of GeneSpring. A two-fold ratio between genes over or under expressed in the presence of uranium and sulfate, relative to the expression values obtained for pyruvate fermentation, was used to create gene lists. Overlap and exclusion between these gene lists were analyzed using Venn diagrams.

### Analytical Methods

Protein from a pelleted culture was extracted by incubation at 95°C for 10 min in 0.1% Triton X-100, 0.1% SDS, 10 mM EDTA and 1 mM Tris-HCl. After a 100-fold dilution, the protein concentration was quantified using the Bradford assay (Biorad, Hercules, CA). Uranium was analyzed by kinetic phosphorescence analysis (KPA) (KPA-11A; Chemcheck Instruments, Richland, WA) after anaerobic filtration (Millipore Millex-GV PVDF 0.2 $\mu$m) and dilution in aerobic 0.1 M $HNO_3$. This measurement is intended to target only U(VI) in solution because U(IV) is expected to be insoluble and retained by filtration. However, if soluble U(IV) (operationally defined as passing through a 0.2 $\mu$m filter) is present in the samples, the addition of aerobic $HNO_3$ after filtration would re-oxidize U(IV) to U(VI), artificially inflating U(VI) concentrations and masking reduction.

Thus, to selectively target soluble U(VI), the measurements were carried out also under anaerobic conditions. Anaerobic U(VI) analyses were carried out by purging all solutions with $N_2$ prior to use and preparing the samples inside the anaerobic glove box and keeping them anaerobic until KPA analysis. Sulfate was analyzed by ion chromatography (DX-500, Dionex, Sunnyvale CA) in an IonPac AS12A column and a 30 mM bicarbonate eluent after filtration and 20-fold dilution.

## RESULTS AND DISCUSSION

### Growth in the Presence of U(VI) or Using Sulfate as an Electron Acceptor

The fermentative growth profiles of *D. reducens* in the presence or absence of U(VI) were remarkably similar (Figure 1A). The increase in biomass recorded by $OD_{600}$ was observed between 10 and 44 h of incubation and corresponded to the exponential growth phase. Subsequently, the $OD_{600}$ values decreased indicating the formation of spores (Junier et al. 2009). During exponential growth, aerobic measurement of solution U(VI) suggested that only 3 $\mu$M of the U(VI) supplied was removed. The vast majority of the reduction was observed after growth had ceased and presumably sporulation had taken place, which is consistent with previous findings linking spores to U(VI) reduction (Junier et al. 2009). RNA was collected at three time points in the presence and absence of U(VI): exponential phase

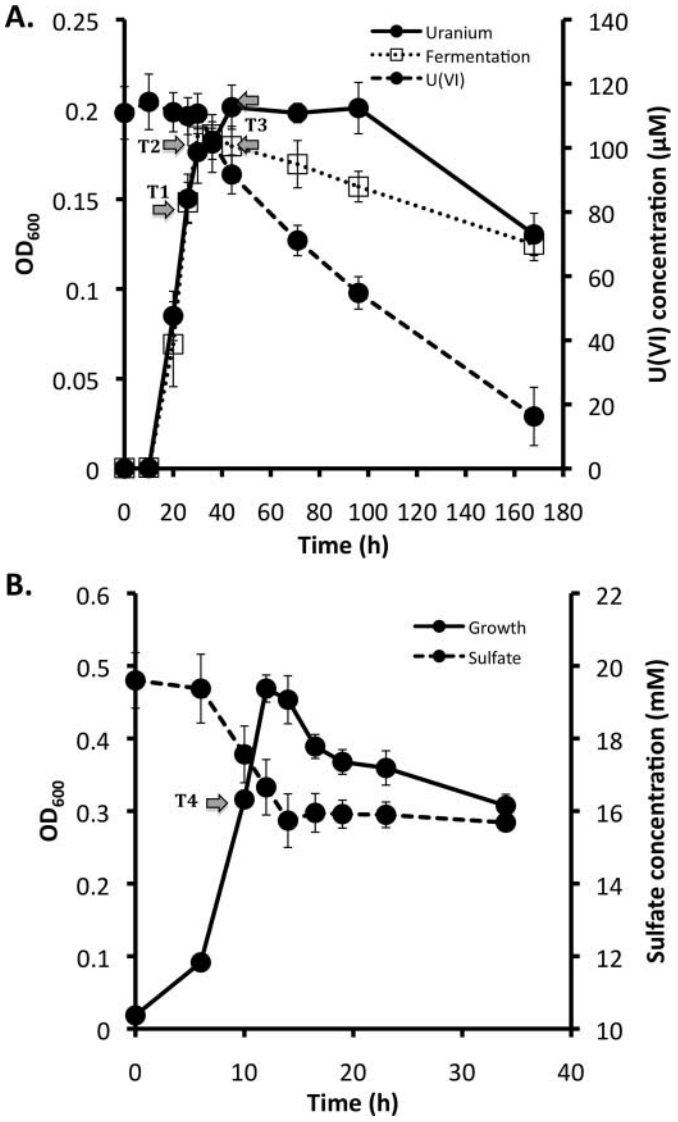


FIG. 1. Growth of *Desulfotomaculum reducens* in the presence and absence of U(VI) and sulfate. A. Growth by pyruvate fermentation in the presence (uranium) and absence (fermentation) of U(VI). U(VI) corresponds to soluble U(VI). B. Growth via sulfate respiration with pyruvate as an electron donor. RNA samples were collected at the time points indicated by the arrows. All values are an average of four biological replicates. Error bars show the standard deviation of the results. T1 = 26 h, T2 = 30 h, T3 = 44 h, and T4 = 10 h.

(T1 = 26 h), late-exponential phase (T2 = 30 h), and before growth ceased (T3 = 44 h).

Growth using sulfate as a terminal electron acceptor and pyruvate as an electron donor was also recorded (Figure 1B). An increase in biomass was observed after 6 h of incubation and the active growth phase lasted until 12–14 h. At the end of the exponential growth phase, a considerably higher cell density was reached in the sulfate system as compared to fermentative growth. A single RNA sample was collected during the exponential phase (T4 = 10 h). At that point 2 mM of sulfate had been reduced (from a total of 3.9 mM after 36 h of incubation).

## Upregulated Genes Common to Uranium and Sulfate Conditions

The expression levels obtained during the exponential phase for the three data sets generated (fermentation labeled as *fermentation*, fermentation and uranium labeled as *uranium* and sulfate reduction labeled as *sulfate*) were compared in order to identify genes differentially expressed in the *uranium* and *sulfate* cases relative to fermentation. A total of 141 genes were differentially expressed in both *uranium* or *sulfate* conditions. Those genes with known function and whose expression was upregulated by the presence of uranium and sulfate were analyzed in detail (Table 1). Several gene clusters involved in energy metabolism were upregulated in the presence of both electron donors. Two clusters of genes annotated as heterodisulfide reductase (Hdr) (dred_0356 to dred_0359 and dred_1325 to dred_1330) were among those. Interestingly, one of the clusters was more highly upregulated in the case of *uranium* (dred_0356 to dred_0359, upregulated up to 7 fold in *uranium* versus 2 fold in *sulfate*), while the other was more highly upregulated in *sulfate* (especially the ORFs dred_1328 to dred_1330).

In the *D. reducens* genome, there are a number of *hdr* genes organized in seven loci (Junier et al. 2010a). The two clusters upregulated here correspond to loci III and IV, which include genes encoding the three subunits of Hdr (*hdr*C, *hdr*B and *hdr*A), and in the case of locus IV, an additional *hdr*A gene followed by the delta subunit of a NiFe hydrogenase (*mvhD*) (Junier et al. 2010a). Hdr proteins play a key role in the energy metabolism of methanogenic archaea.

Initially, it was thought that Hdr was unique to methanogens, but genes encoding homologous proteins were identified in a broad range of microorganisms unable to perform methanogenesis (Hedderich et al. 1998). Recently, Hdr proteins have been implicated in electron transfer during sulfate reduction in the Gram-negative deltaproteobacterium *Desulfobacterium autotrophicum* HRM2 (Strittmatter et al. 2009). The upregulation of these genes during sulfate reduction in *D. reducens* is consistent with a role in that metabolism. However, upregulation during exposure to U(VI) is more revealing as it points to a potential transcriptomic response that involves reduction activity rather than solely a general toxicity response.

This observation is not isolated: another gene that was highly upregulated in the presence of U(VI) (over 10-fold) as well as during sulfate reduction (over 7-fold) was identified as an iron-sulfur cluster-binding protein (dred_0490, Table 1). A BLASTP search for this ORF revealed a conserved domain corresponding to a polyferredoxin. Thus, this putative ferredoxin gene that is presumably involved in electron transfer reactions is upregulated in the presence of U(VI).

Furthermore, a key component of the respiratory chain, the NADH-quinone oxidoreductase (NADH-QOR) (dred_2036 to dred_2046), was also upregulated in both systems. The upregulation was not uniform for the *sulfate* and *uranium* cases. Higher upregulation was observed in most of the subunits in the case of *uranium*. For example, two subunits (dred_2045 and dred_2046)

TABLE 1
Selected genes involved in energy metabolism upregulated in mid-exponential phase both for cultures growing fermentatively with pyruvate (T1 = 26 h) in the presence of U(VI) (uranium) as well as cultures growing by sulfate respiration (T4 = 10 h) with pyruvate as an electron donor (sulfate)

| | Fold-change | | |
|---|---|---|---|
| Gene Name | Uranium | Sulfate | Description |
| dred_0356 | 4.64 | <2 | heterodisulfide reductase, subunit A |
| dred_0357 | 7.56 | 2.01 | heterodisulfide reductase, subunit B |
| dred_0358 | 7.79 | 2.12 | heterodisulfide reductase subunit C |
| dred_0359 | 3.77 | 0.30 | redox-active disulfide protein 2 |
| dred_0490 | 10.48 | 7.80 | iron-sulfur cluster-binding protein |
| dred_1325 | 2.64 | 2.56 | heterodisulfide reductase, C subunit |
| dred_1326 | 3.14 | 3.58 | heterodisulfide reductase, B subunit |
| dred_1327 | 3.94 | 9.36 | heterodisulfide reductase, A subunit |
| dred_1328 | <2 | 21.97 | heterodisulfide reductase, subunit A |
| dred_1329 | <2 | 29.64 | methyl viologen-reducing hydrogenase, subunit delta |
| dred_1330 | <2 | 21.15 | 4Fe-4S binding domain protein |
| dred_1502 | 2.02 | 2.42 | carbon monoxide dehydrogenase medium chain |
| dred_1503 | <2 | <2 | carbon monoxide dehydrogenase small chain |
| dred_1504 | <2 | 2.78 | aerobic-type carbon monoxide dehydrogenase, large subunit |
| dred_1654 | 5.31 | 3.08 | hydrogenase |
| dred_1655 | 6.32 | 3.35 | NADH:ubiquinone oxidoreductase, NADH-binding (51 kD) subunit |
| dred_1656 | 7.52 | 3.95 | Fe-hydrogenase gamma subunit |
| dred_2036 | <2 | 4.06 | Proton-translocating NADH-quinone oxidoreductase, chain N |
| dred_2037 | <2 | 5.47 | proton-translocating NADH-quinone oxidoreductase, chain M |
| dred_2038 | 4.29 | 3.95 | proton-translocating NADH-quinone oxidoreductase, chain L |
| dred_2039 | 8.98 | 4.99 | NADH-quinone oxidoreductase, K subunit |
| dred_2040 | 11.31 | 4.51 | NADH-quinone oxidoreductase, chain 6 |
| dred_2041 | 15.48 | 3.85 | NAD(P)H-quinone oxidoreductase subunit I |
| dred_2042 | 22.73 | 5.16 | NADH-quinone oxidoreductase, H subunit |
| dred_2043 | 12.47 | 2.11 | NADH dehydrogenase I, D subunit |
| dred_2044 | 17.71 | 2.09 | NADH dehydrogenase, subunit C |
| dred_2045 | 16.36 | <2 | NADH-quinone oxidoreductase, B subunit |
| dred_2046 | 16.26 | <2 | NAD(P)H-quinone oxidoreductase chain 3 |
| dred_2097 | 5.04 | 8.96 | manganese-dependent inorganic pyrophosphatase |
| dred_2228 | 2.11 | 5.51 | 4Fe-4S ferredoxin, iron-sulfur binding protein |

Expression levels are relative to cultures grown fermentatively with pyruvate.



were over 16-fold upregulated in *uranium*, while they were below the 2-fold upregulation threshold in sulfate. Conversely, chains N and M (dred_2036 and dred_2037) of the protein were more highly upregulated in the presence of sulfate than that of uranium. NADH-QOR is the enzyme that couples the oxidation of NADH and the transfer of electrons down the electron transport chain to extra-cytoplasmic proton translocation, an energy-generating process (Brandt 2006).

NADH-QOR is associated with respiratory processes (such as sulfate reduction) but not typically with fermentative processes that do not require proton translocation since energy is generated via substrate-level phosphorylation. Thus, the upregulation of NADH-QOR during pyruvate fermentation in the presence of U(VI) (as compared to in its absence) is indicative of the fact that electrons are shuttled through the electron transport chain, presumably to allow U(VI) reduction.

In prokaryotes, NADH-QOR generally consists of 14 subunits. An analysis of the genome of *D. reducens* reveals that only 11 of those subunits can be identified in the cluster of genes upregulated in the presence of sulfate and uranium. The three missing subunits (referred to as 75 kDa, 51 kDa and 24 kDa subunits) constitute the N module of the enzyme, which is implicated in the oxidation of NADH (Brandt, 2006). Interestingly, three genes annotated in the genome as trimeric hydrogenases (dred_1654 to dred_1656) (Junier et al. 2010a) were also upregulated in both *sulfate* and *uranium* conditions (Table 1).

A closer analysis of the annotation of these three genes reveals that they contain all the domains involved in the function

of the NADH-QOR N module proteins, including several iron-sulfur- or NADH-binding regions as well as an NADH dehydrogenase domain. More importantly, the three subunits are homologues to the missing 75 kDa (dred_1654), 51 kDa (dred_1655) and 24 kDa (dred_1656) subunits. We hypothesize that these three genes could interact with the other two modules of the NADH oxidoreductase in order to transfer electrons from NADH to the respiratory chain and to form a functional NADH-QOR. The function of the hydrogenase domain present in locus dred_1654 is unknown.

The gene expression profiles suggest that U(VI) is being reduced by vegetative cells despite no measurable removal of U(VI) from solution by aerobic KPA measurements at the RNA sampling time-points. This is a crucial point because, based on the growth and reduction curves obtained here (Figure 1), as well as in previous experiments (Junier et al. 2009; Junier et al. 2010b), U(VI) reduction by vegetative cells was determined to be minimal during active fermentative growth with pyruvate.

In order to reconcile U(VI) reduction data with the expression data, we evaluated the possibility that U(IV) produced by vegetative cells during fermentative growth is nominally soluble and not removed by filtration and is measured as U(VI) via aerobic KPA measurements. We repeated the growth experiment and, this time, measured the filtrate both aerobically and anaerobically for U(VI) to differentiate between nominally soluble (passes through 0.2 $\mu$m filter) U(IV) and U(VI) (Figure 2). The results show a clear discrepancy between the anaerobic and aerobic measurements during exponential growth and after growth has ceased. We conclude that, during the exponential growth phase, U(IV) indeed passes through the filter and is measured as U(VI) when the analysis is done aerobically. Thus, a higher fraction of total U(VI) is being reduced by vegetative cells than determined by strictly aerobic KPA measurements. Vegetative cells reduce U(VI) but at a rate that is considerably slower than the rate of reduction by spores (Junier et al. 2009). Thus, the sharp increase in the reduction rate during the stationary phase (Figure 2) is consistent with the involvement of spores in U(VI) reduction as was previously shown (Junier et al. 2009).

Despite the difference in the timeframe between the two experiments (Figures 1 and 2), a comparison of U(VI) reduction at corresponding points in the exponential growth curve (indicated by an arrow in Figure 2) shows significantly more U(VI) reduction when the measurement is done anaerobically. This suggests that indeed a measurable fraction of U(VI) had been reduced at the time the RNA samples were collected and resolves the apparent contradiction between U(VI) reduction and expression data.

## Genes Upregulated Exclusively in the Presence of U(VI)

A comparison of the genes upregulated in the *uranium* but not in the *sulfate* case lead to the identification of 176 genes. This group included genes involved in energy metabolism, heavy metal resistance, and iron metabolism (Table 2).

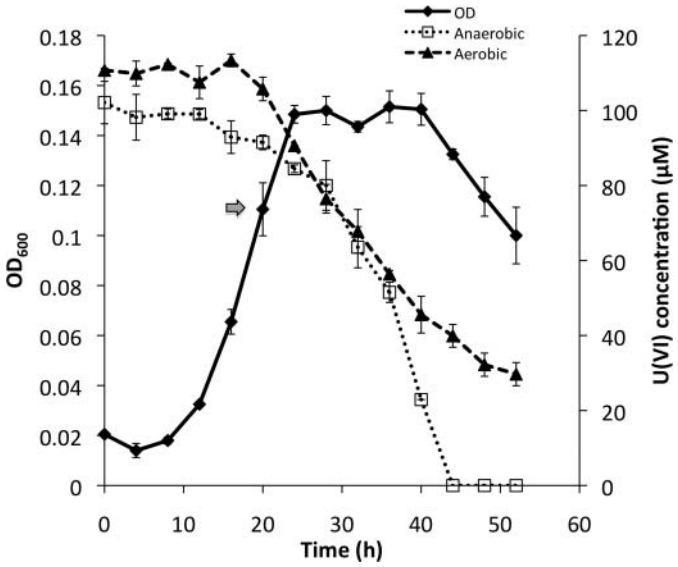


FIG. 2. Growth of *Desulfotomaculum reducens* in the presence of U(VI). U(VI) was measured aerobically (aerobic) and anaerobically (anaerobic). For this experiment only 10 mM pyruvate was used as electron donor for 100 $\mu$M of U(VI). An arrow indicates the equivalent physiological state analyzed for T1 in Figure 1.

Two electron-transfer flavoproteins (dred_1538 and dred_1539) were upregulated in the presence of U(VI). The upregulation of flavoproteins, which are enzymes commonly involved in electron transfer during respiration, gives additional support to the finding that vegetative cells catalyze the reduction of U(VI).

A gene cluster annotated as a trimeric [FeFe] hydrogenase (dred_1651 to dred_1653) (distinct from the one upregulated in both the *uranium* and *sulfate* cases) is highly upregulated in the presence of U(VI). This is significant because hydrogen was identified as the electron donor for U(VI) reduction in spores of *D. reducens* (Junier et al. 2009). However, the function of this trimeric hydrogenase should be considered cautiously since it possesses the same gene structure and is contiguous to the other cluster that was annotated as a trimeric hydrogenase (dred_1654–6 in Table 1) but suggested to be part of the NADH-QOR complex (see previous section). Unveiling the precise role and function of these two hydrogenases and their involvement in U(VI) reduction by vegetative cells warrants future investigation.

Two regions containing genes putatively implicated in *c*-type cytochrome biogenesis (dred_1527 to dred_1533 and dred_0545 to dred_0554) were upregulated in the presence of uranium. One of the regions (dred_1527 to dred_1533) includes genes involved in heavy metal resistance such as cadmium efflux pumps, a ferric iron ATP-binding cassette (ABC) transporter, a thioredoxin (dred_1533), and a cytochrome *c* biogenesis protein that contains six transmembrane domains. The second region (dred_0545 to dred_0554) contains several hypothetical proteins localized in the vicinity of a permease (dred_0548), a putative small redox

TABLE 2
Selected genes upregulated in mid-exponential phase (T1 = 26 h) cultures grown fermentatively with pyruvate in the presence of U(VI) but not upregulated in cultures grown on sulfate as an electron acceptor

| Gene Name | Fold-change | Description |
|---|---|---|
| Energy metabolism | | |
| dred_1538 | 2.58 | electron transfer flavoprotein, beta subunit |
| dred_1539 | 2.27 | electron transfer flavoprotein, alpha subunit |
| dred_1651 | 11.19 | hydrogenase |
| dred_1652 | 8.91 | NADH:ubiquinone oxidoreductase, NADH-binding (51 kD) subunit |
| dred_1653 | 9.35 | Fe-hydrogenase gamma subunit |
| c-type cytochrome biogenesis | | |
| dred_0545 | 3.68 | hypothetical protein |
| dred_0546 | 3.77 | redox-active disulfide protein 2 |
| dred_0547 | 3.76 | DNA-binding protein |
| dred_0548 | 8.50 | permease |
| dred_0549 | 4.85 | hypothetical protein |
| dred_0550 | 4.34 | hypothetical protein |
| dred_0551 | 6.77 | cytochrome c biogenesis protein, transmembrane region |
| dred_0552 | 4.69 | hypothetical protein |
| dred_0553 | 5.34 | DGC domain family |
| dred_0554 | 6.38 | sodium/bile acid symporter family protein |
| dred_1527 | 5.12 | cadmium-translocating P-type ATPase |
| dred_1528 | 4.99 | cadmium efflux system accessory protein |
| dred_1529 | 2.79 | ferric iron uptake ABC transporter |
| dred_1530 | 5.76 | ABC-type Fe3+ transport system, permease component |
| dred_1531 | 5.38 | ABC transporter, ATP-binding protein |
| dred_1532 | 3.76 | cytochrome c biogenesis protein, transmembrane region |
| dred_1533 | 4.07 | thioredoxin |
| Iron metabolism | | |
| dred_0647 | 2.57 | ferrous ion uptake protein A |
| dred_0661 | 4.59 | ferrous iron transport protein B |
| dred_0662 | 6.69 | ferrous ion uptake protein A |
| dred_2644 | 3.06 | Transcriptional regulator, Fur family |
| dred_2718 | 6.21 | ferrous iron transport protein A, FeoA |
| dred_2719 | 12.15 | ferrous iron transport protein B |
| dred_2720 | 10.18 | ferrous iron transport protein B, FeoB |
| dred_2721 | 8.16 | iron dependent repressor |
| dred_2722 | 5.56 | hypothetical protein |
| dred_2723 | 10.79 | ferrous iron transport protein B, FeoB |
| dred_2725 | 3.46 | possible ferrous iron transport protein |
| dred_2726 | 3.55 | methyl-accepting chemotaxis protein, putative |
| dred_2727 | 6.55 | HDIG domain protein |
| Genes involved in heavy metal resistance | | |
| dred_0555 | 6.56 | DGC domain containing protein |
| dred_0556 | 4.87 | permease |
| dred_0557 | 3.03 | hypothetical protein |
| dred_0558 | 3.89 | arsenical-resistance protein |
| dred_0559 | 3.33 | transcriptional regulator, ArsR family |
| dred_0561 | 2.73 | transcriptional regulator, ArsR family |
| dred_0562 | 2.59 | hypothetical protein |
| dred_1119 | 5.30 | sodium-dependent transporter |
| dred_2140 | 2.62 | copper-translocating P-type ATPase |
| dred_2141 | 3.15 | conserved hypothetical protein |
| dred_2645 | 2.37 | ABC-type Zn2+/Mn2+ transport system, Zn-binding periplasmic component |
| dred_2646 | 2.07 | ABC-type Zn2+/Mn2+ transport system, ATPase component |
| dred_2982 | 2.34 | copper-translocating P-type ATPase |

Expression levels are relative to cultures grown fermentatively with pyruvate.

active disulfide protein (dred_0546) and a cytochrome *c* biogenesis protein (dred_0551), which according to bioinformatic predictions is an integral membrane protein with six transmembrane domains. Thus, two genes annotated as encoding membrane-spanning *c*-type cytochrome biogenesis proteins (dred_0551 and dred_1532) are upregulated in the presence of uranium but not sulfate. However, two other *c*-type cytochrome biogenesis proteins (dred_0702-3) associated with the only *c*-type cytochrome present in the genome (NrfHA) are not differentially expressed.

The putative small redox active disulfide protein (dred_0546) was analyzed by BLAST with the Protein Data Bank (http://www.rcsb.org/pdb) and the best hit obtained was the thioredoxin of the archaeon *Methanobacterium thermoautotrophicum* (E-value of 1.8E-10). A pBLAST search also revealed the presence of a thioredoxin-like superfamily domain in this ORF. Very recently, it was determined that a cytoplasmic thioredoxin, a thioredoxin reductase and an associated oxidoreductase participate in U(VI) reduction in the cytoplasm of the SRB *Desulfovibrio desulfuricans* G20 (Li and Krumholz, 2009). Besides the two thioredoxins (dred_1533 and dred_0546) that were mentioned above as being upregulated in the presence of uranium, there are three additional ORFs annotated as thioredoxins in the *D. reducens* genome (dred_0762, dred_0904 and dred_2669). One of those (dred_2669) is associated with a thioredoxin reductase (dred_2670) (Junier et al. 2010a). However, none of these four ORFs (dred_0762, dred_0904, dred_2669 and dred_2670) displayed differential expression in the presence of uranium. Thus, the two upregulated thioredoxin-like proteins (dred_1533 and dred_0546) are proposed to be implicated in *c*-type cytochrome biogenesis based on their proximity to genes (dred_0551 and dred_1533) encoding membrane-spanning *c*-type cytochrome biogenesis proteins, the upregulation of an entire gene cluster that includes a metal transporter (dred_0554, dred_1527-8, dred_1529-31) as well as the similarity of the protein encoded by dred_1533 to CcmG, a cytochrome maturation protein as determined by BLASTP (E-value = 3e-28). The data available point to the involvement of some thioredoxins in cytochrome synthesis but are insufficient to allow us to comment on their direct role in U(VI) reduction.

The observations described above suggest that the presence of uranium enhances the synthesis of *c*-type cytochromes. While this type of protein has been implicated in U(VI) reduction in other bacteria (Bencheikh-Latmani et al. 2005; Shelobolina et al. 2007), there is no direct evidence of its role in *D. reducens*. A sole *c*-type cytochrome was identified in the genome of *D. reducens*. It is annotated as a cytochrome $c_{552}$ nitrite reductase (NrfHA) and is encoded by the genes dred_0700 and dred_0701 (Junier et al. 2010a). Neither gene nor the two cytochrome biogenesis-related genes associated with those two (dred_0702-3) were upregulated in the presence of uranium. Therefore, the involvement of NrfHA in U(VI) reduction remains unproven.

Accordant with evidence for the expression of *c*-type cytochrome biogenesis proteins in response to U(VI) is the observation that genes involved in iron transport, including various ferrous iron uptake proteins (dred_647, dred_0661 to dred_0662, and dred_2718 to dred_2727) and a transcriptional regulator involved in Fe homeostasis (dred_2644), were upregulated in the presence of uranium (Table 2). Iron homeostasis is essential for the survival of bacteria but, under aerobic condition, the poor solubility of iron hinders its uptake. However, under the present anaerobic conditions, ferrous iron is available, and the activation of the ferrous iron transport system, FeoAB, matches the expectation of the presence of ferrous rather than ferric iron. The upregulation of *feoAB* genes is also in line with a higher requirement for iron due to the synthesis of Fe-bearing proteins such as cytochromes.

However, the upregulation of iron transporters can also be in response to stress induced by uranium. In *S. oneidensis*, the stress response to chromium and strontium lead to the upregulation of proteins involved in ferric iron transport, specifically siderophore biosynthesis proteins and a siderophore-mediated transport protein, TonB (Brown et al. 2006a, 2006b) as well as the global regulator for ferric uptake (Fur) (Thompson et al. 2002). TonB receptors were also upregulated in response to uranium stress in *C. crescentus* under aerobic conditions (Hu et al. 2005).

A metal toxicity response is discernable in *D. reducens* as well. Several genes involved in heavy metal resistance were upregulated in the presence of uranium (Table 2). These include genes involved in arsenic resistance (dred_0555 to dred_0562) and copper-translocation (dred_2140 and dred_2982). The upregulation of two transcriptional regulators from the ArsR family (dred_0559 and dred_0561), as part of an operon (dred_ 0555 to dred_0562) that also contains an arsenical-resistance protein (see Table 2), is significant since those repressors belong to a family of transcriptional repressors (SmtB/ArsR family) that regulates the expression of operons linked to stress-inducing concentrations of heavy metal ions (Busenlehner et al. 2003). Also, genes for $Zn^{+2}/Mn^{+2}$ transport systems (dred_2645 and dred_2646) were up-regulated in the presence of U(VI). It is unclear whether those genes are involved in efflux or in uptake. If the latter is true, these genes would not be implicated in a mechanism of stress response to heavy metals.

Analysis of genes upregulated in the uranium case but not in the sulfate case reveals the importance of metal detoxification, the induction of *c*-type cytochrome synthesis and the increased uptake of iron but shows no evidence of upregulation of the sole *c*-type cytochrome encoded in the genome: NrfHA.

## Time-Dependent Changes in Gene Expression

In addition to the results from the mid-exponential phase time point discussed in the previous sections, changes in gene expression in cells of *D. reducens* were analyzed for two more time points (Figure 1). The genes whose expression exceeded the activity threshold for all three time points and at least one condition *-fermentation* or *uranium-* (1902 genes out of 3,258 genes) were clustered according to their expression profile (Figure 3).

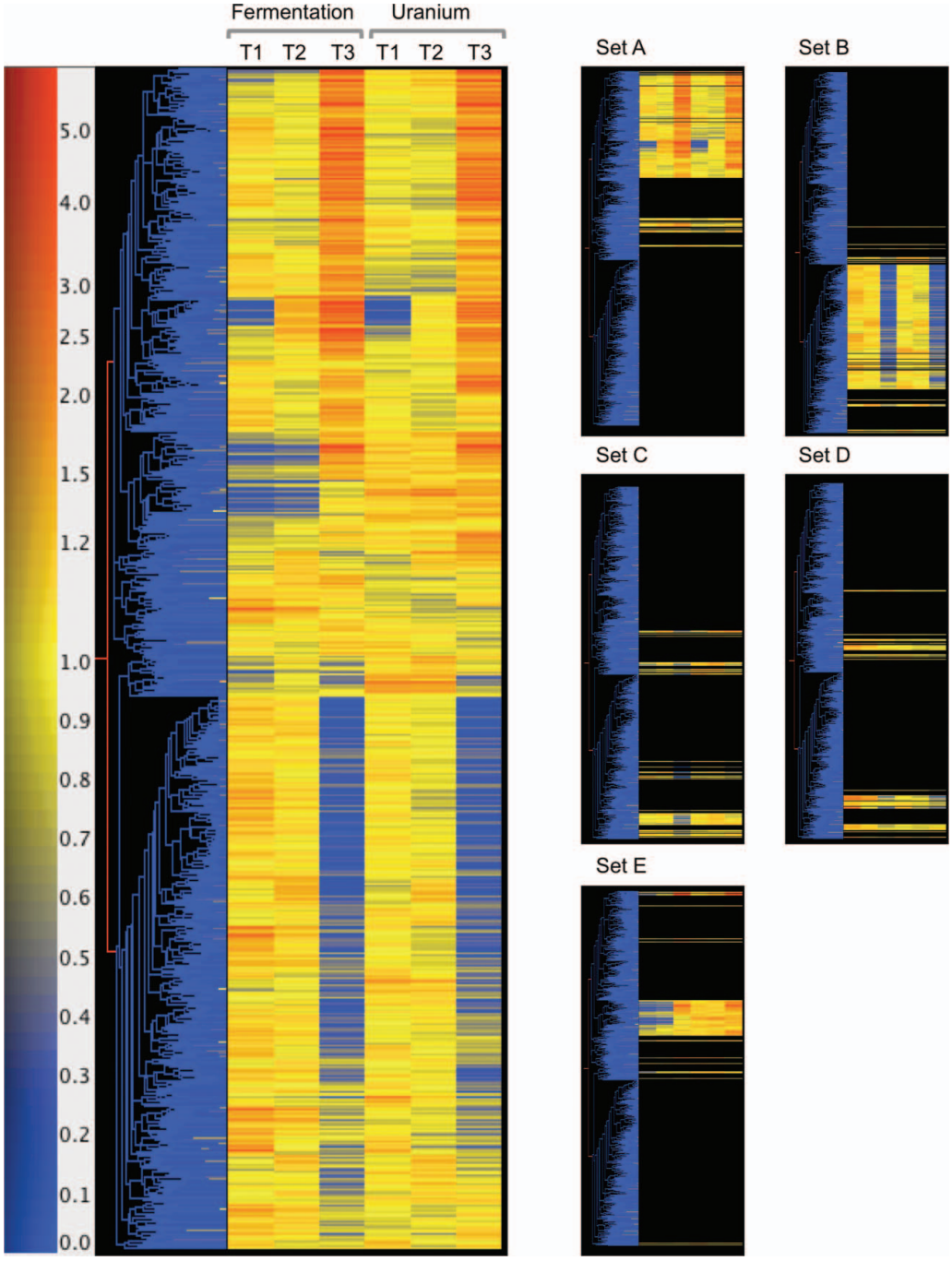


FIG. 3. K-mean clustering of genes in the expression profiles during growth by pyruvate fermentation in the presence (uranium) and absence (fermentation) of uranium. The red color represents the levels of induction, while the blue color represents the levels of repression. T1 = 26 h, T2 = 30 h, T3 = 44 h (color figure available online).

Five sets of genes could be distinguished based on the expression patterns. Among those, sets A and B contained the majority of the genes (1291 genes). The expression of the genes in those two sets was dominated by a significant change in expression profiles at T3 (44 h), both for up- (set A) and downregulation (set B) relative to the earlier time points (T1 and T2). This dramatic change in the expression profile coincided with evidence of RNA degradation as well as a decrease in protein content in the culture pellets (data not shown). Both findings, a decrease in RNA quality and in protein content, are indicative of the sporulation of the cultures after pyruvate is consumed (Junier et al. 2009). Sporulation may be the dominant process taking place at this time point, as evidenced by the fact that the expression profiles for T3 in the presence and absence of U(VI) cluster

TABLE 3
Selected genes upregulated at T2 (30 h) and T3 (44 h) versus T1 (26 h) in cultures in the presence of uranium (*Uranium*) or in its absence (*Fermentation*)

| | Fold Change | | |
|---|---|---|---|
| Gene Name | Uranium | Fermentation | Description |
| T2 versus T1 | | | |
| dred_0118 | 11.89 | 6.99 | sporulation protein YabP |
| dred_0119 | 14.18 | 6.76 | spore cortex biosynthesis protein YabQ, putative |
| dred_0265 | 20.83 | 15.15 | germination-specific N-acetylmuramoyl-L-alanine amidase |
| dred_0686 | 2.22 | 2.00 | RNA polymerase sigma-E factor |
| dred_1056 | 3.34 | 2.75 | stage III sporulation protein AA |
| dred_1057 | 2.79 | 2.55 | stage III sporulation protein AB |
| dred_1058 | 2.60 | 2.27 | stage III sporulation protein AC |
| dred_1059 | 3.11 | 2.73 | stage III sporulation protein AD |
| dred_1060 | 3.08 | 2.57 | stage III sporulation protein AE |
| dred_1061 | 3.47 | 3.41 | stage III sporulation protein AF, putative |
| dred_1062 | 3.39 | 3.04 | stage III sporulation protein AG, putative |
| dred_1063 | 3.69 | 3.24 | stage III sporulation protein AH |
| dred_1163 | 3.56 | 3.33 | stage IV sporulation protein A |
| dred_2005 | 2.16 | | sporulation protein YhbH |
| dred_2006 | 2.23 | 2.34 | serine protein kinase PrkA |
| dred_2485 | 8.77 | 5.43 | sporulation protein YqfD |
| dred_2503 | 9.62 | 7.35 | stage II sporulation protein P |
| dred_2539 | 5.41 | 4.90 | peptidase M50 |
| dred_2540 | 11.51 | 6.49 | peptidase M23B |
| dred_3146 | 4.37 | 5.49 | hypothetical protein |
| dred_3147 | 2.11 | 2.40 | stage II sporulation protein D |
| dred_3173 | 5.35 | 5.08 | cell-wall endopeptidase, M23B family |
| dred_3314 | 24.81 | 12.39 | putative sporulation protein YyaC |
| T3 versus T1 | | | |
| dred_0118 | 42.32 | 52.64 | sporulation protein YabP |
| dred_0119 | 31.13 | 56.37 | spore cortex biosynthesis protein YabQ, putative |
| dred_0265 | 53.51 | 66.99 | germination-specific N-acetylmuramoyl-L-alanine amidase |
| dred_1063 | 8.096 | 7.957 | stage III sporulation protein AH |
| dred_2485 | 13.22 | 24.47 | sporulation protein YqfD |
| dred_2503 | 25.87 | 34.64 | stage II sporulation protein P |
| dred_2539 | 8.529 | 12.88 | peptidase M50 |
| dred_2540 | 11.95 | 32.48 | peptidase M23B |
| dred_3147 | 8.369 | 7.693 | stage II sporulation protein D |
| dred_3173 | 30.15 | 24.53 | cell-wall endopeptidase, M23B family |
| dred_3314 | 34.82 | 82.12 | putative sporulation protein YyaC |



together (data not shown) whereas the profiles for earlier time points do not.

In addition, a number of sporulation-related genes were highly upregulated both at T2 and T3 relative to T1. As the change in expression for T3 vs. T1 was large for many genes, only genes up-or down-regulated 8-fold or higher were considered for that time point (Table 3). There are clear differences in the expression profiles of sporulation-related genes at T3 as compared to T2, which is consistent with the evidence presented earlier. However, the limited time resolution of the data precludes the detailed analysis of the subtleties of gene expression during *D. reducens* sporulation but allows a few observations.

As expected from the gene expression profile clustering (Figure 3), a similar set of genes was differentially expressed in the presence and absence of uranium. This suggests that sporulation is independent of the presence of uranium and occurs as

TABLE 4
Selected genes upregulated in mid-exponential (T1 = 26 h) and late exponential (T2 = 30 h) phase cultures grown fermentatively with pyruvate in the presence of U(VI)

| | Fold-change | | |
|---|---|---|---|
| Gene Name | T1 | T2 | Description |
| Energy metabolism | | | |
| dred_0356 | 4.64 | <2 | heterodisulfide reductase, subunit A |
| dred_0357 | 7.56 | 4.49 | heterodisulfide reductase, subunit B |
| dred_0358 | 7.79 | 5.71 | heterodisulfide reductase subunit C |
| dred_0359 | 3.77 | <2 | redox-active disulfide protein 2 |
| dred_0490 | 10.48 | 7.11 | iron-sulfur cluster-binding protein |
| dred_1325 | 2.64 | <2 | heterodisulfide reductase, C subunit |
| dred_1326 | 3.14 | 2.67 | heterodisulfide reductase, B subunit |
| dred_1327 | 3.94 | 4.45 | heterodisulfide reductase, A subunit |
| dred_1328 | <2 | <2 | heterodisulfide reductase, subunit A |
| dred_1329 | <2 | <2 | methyl viologen-reducing hydrogenase, subunit delta |
| dred_1330 | <2 | <2 | 4Fe-4S binding domain protein |
| dred_1538 | 2.58 | <2 | electron transfer flavoprotein, beta subunit |
| dred_1539 | 2.27 | 2.16 | electron transfer flavoprotein, alpha subunit |
| dred_1651 | 11.19 | 4.62 | hydrogenase |
| dred_1652 | 8.91 | 2.68 | NADH:ubiquinone oxidoreductase, NADH-binding (51 kD) subunit |
| dred_1653 | 9.35 | 4.49 | Fe-hydrogenase gamma subunit |
| dred_1654 | 5.31 | 2.92 | hydrogenase |
| dred_1655 | 6.32 | 3.83 | NADH:ubiquinone oxidoreductase, NADH-binding (51 kD) subunit |
| dred_1656 | 7.52 | 4.40 | Fe-hydrogenase gamma subunit |
| dred_2038 | 4.29 | <2 | proton-translocating NADH-quinone oxidoreductase, chain L |
| dred_2039 | 8.98 | 7.84 | NADH-quinone oxidoreductase, K subunit |
| dred_2040 | 11.31 | 11.79 | NADH-quinone oxidoreductase, chain 6 |
| dred_2041 | 15.48 | 13.48 | NAD(P)H-quinone oxidoreductase subunit I |
| dred_2042 | 22.73 | <2 | NADH-quinone oxidoreductase, H subunit |
| dred_2043 | 12.47 | 7.71 | NADH dehydrogenase I, D subunit |
| dred_2044 | 17.71 | 10.57 | NADH dehydrogenase, subunit C |
| dred_2045 | 16.36 | 9.70 | NADH-quinone oxidoreductase, B subunit |
| dred_2046 | 16.26 | 12.30 | NAD(P)H-quinone oxidoreductase chain 3 |
| c-type cytochrome biogenesis | | | |
| dred_0545 | 3.68 | 2.12 | hypothetical protein |
| dred_0546 | 3.77 | 2.52 | redox-active disulfide protein 2 |
| dred_0547 | 3.76 | 4.69 | DNA-binding protein |
| dred_0548 | 8.50 | 4.58 | permease |
| dred_0549 | 4.85 | 3.03 | hypothetical protein |
| dred_0550 | 4.34 | 2.95 | hypothetical protein |
| dred_0551 | 6.77 | 4.08 | cytochrome c biogenesis protein, transmembrane region |
| dred_0552 | 4.69 | 3.70 | hypothetical protein |
| dred_0553 | 5.34 | 3.41 | DGC domain family |
| dred_0554 | 6.38 | 4.04 | sodium/bile acid symporter family protein |
| dred_1527 | 5.12 | 3.76 | cadmium-translocating P-type ATPase |
| dred_1528 | 4.99 | 4.61 | cadmium efflux system accessory protein |
| dred_1529 | 2.79 | 3.87 | ferric iron uptake ABC transporter |
| dred_1530 | 5.76 | 3.99 | ABC-type Fe3+ transport system, permease component |
| dred_1531 | 5.38 | 2.07 | ABC transporter, ATP-binding protein |
| dred_1532 | 3.76 | <2 | cytochrome c biogenesis protein, transmembrane region |
| dred_1533 | 4.07 | <2 | thioredoxin |

*(Continued on next page)*

TABLE 4
Selected genes upregulated in mid-exponential (T1 = 26 h) and late exponential (T2 = 30 h) phase cultures grown fermentatively with pyruvate in the presence of U(VI) *(Continued)*

| | Fold-change | | |
|---|---|---|---|
| Gene Name | T1 | T2 | Description |
| Iron metabolism | | | |
| dred_0647 | 2.57 | 2.13 | ferrous ion uptake protein A |
| dred_0661 | 4.59 | 2.34 | ferrous iron transport protein B |
| dred_0662 | 6.69 | 3.54 | ferrous ion uptake protein A |
| dred_2718 | 6.21 | 2.01 | ferrous iron transport protein A, FeoA |
| dred_2719 | 12.15 | 4.62 | ferrous iron transport protein B |
| dred_2720 | 10.18 | 3.85 | ferrous iron transport protein B, FeoB |
| dred_2721 | 8.16 | 3.86 | iron dependent repressor |
| dred_2722 | 5.56 | 3.38 | hypothetical protein |
| dred_2723 | 10.79 | 3.63 | ferrous iron transport protein B, FeoB |
| dred_2725 | 3.46 | <2 | possible ferrous iron transport protein |
| dred_2726 | 3.55 | <2 | methyl-accepting chemotaxis protein, putative |
| dred_2727 | 6.55 | <2 | HDIG domain protein |
| Genes involved in heavy metal resistance | | | |
| dred_0555 | 6.56 | 3.67 | DGC domain containing protein |
| dred_0556 | 4.87 | 3.40 | permease |
| dred_0557 | 3.03 | 3.73 | hypothetical protein |
| dred_0558 | 3.89 | 3.38 | arsenical-resistance protein |
| dred_0559 | 3.33 | 2.55 | transcriptional regulator, ArsR family |
| dred_0561 | 2.73 | <2 | transcriptional regulator, ArsR family |
| dred_0562 | 2.59 | <2 | hypothetical protein |
| dred_1119 | 5.30 | 2.83 | sodium-dependent transporter |
| dred_2140 | 2.62 | 2.57 | copper-translocating P-type ATPase |
| dred_2141 | 3.15 | 2.55 | conserved hypothetical protein |

Expression levels are relative to cultures grown fermentatively with pyruvate.



a result of the depletion of pyruvate (Junier et al. 2009). Genes upregulated at T2 relative to T1 include those encoding the proteins YabP and YabQ (dred_0118 and dred_0119) and the stage IV sporulation protein A (dred_1163), all of which are involved in the assembly of the spore coat in *Bacillus subtilis* (Roels et al. 1992; Stevens et al. 1992; van Ooij et al. 2004). The level of upregulation (relative to T1) of the genes encoding for YabP and YabQ increases significantly at T3 (e.g., 11.9 fold at T2 versus 42.3 fold at T3 in cells grown in the presence of uranium; Table 3) suggesting the continued importance of the spore coat assembly at the latter time point.

The sporulation sigma factor E (dred_0686) -known to be involved in gene expression regulation in the mother cell after asymmetric division- and an operon encoding proteins of the stage III of sporulation (dred_1056 to dred_1063) -whose expression depends from sigma factor E (Illing and Errington, 1991)- were upregulated, but only at T2. The only gene from the stage III sporulation operon that was still upregulated at T3 was the one coding for the protein AH (dred_1063). The stage III sporulation protein AH has been implicated in the engulfment of the forespore during the later stages of spore formation (Broder and Pogliano, 2006). Also, the stage II sporulation proteins P (dred_2503) and D (dred_3147), two of the three major proteins involved engulfment of the forespore in *B. subtilis* (Doan et al. 2005), were upregulated in T2 and T3. At T3, both genes (dred_2503 and dred_3147) were significantly more upregulated than at T2. Other genes upregulated at T2 that belong to the sigma E regulon (Feucht et al. 2003) were the ones encoding the sporulation protein YhbH (dred_2005) and YqfD (dred_2485).

Thus, based on the gene expression profiles, sporulation is underway at T2 (30 h) and still ongoing at T3 (44 h). This apparently rather long time span for sporulation is likely due to the lack of synchronization within the culture leading to overlapping signals between different stages of sporulation.

While there is a clear shift in overall gene expression from T1 to T2 for both the *fermentation* and *uranium* cases, the comparison between fermentation and uranium at T2 is essentially the same as that at T1 (Table 4). All the key genes that were upregulated in the presence of uranium at T1 (e.g., heterodisulfide

reductase, NADH:QOR, *c*-type cytochrome biogenesis, iron transporters) were still upregulated at T2 with the exception of one of the *c*-type cytochromes biogenesis genes and associated thioredoxin gene (dred_1532-3). This suggests that the U(VI) reduction process inferred from the gene upregulated at T1 is still underway later in the growth phase. However, at the onset of the stationary phase (T3), this and probably other processes are superseded by sporulation.

## CONCLUSIONS

In conclusion, the transcriptomic response of *D. reducens* MI-1 to the presence of uranium is consistent with U(VI) reduction by vegetative cells. This metabolic process likely involves proteins from the respiratory chain such as flavoproteins, NADH quinone oxidoreductase and the heterodisulfide reductase. In addition, there is indirect evidence – the upregulation of *c*-type cytochrome synthesis proteins as well as ferrous iron transporters—suggesting the likely involvement of *c*-type cytochromes in U(VI) reduction but no direct evidence for the upregulation of the sole *c*-type cytochrome present in the genome of *D. reducens*. Changes in gene expression overtime show the activation of genes mainly related to sporulation at the onset of the stationary phase, which coincided with the formation of spores after the depletion of the electron donor.

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