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Transcriptome Profiling of *Shewanella oneidensis* Gene Expression following Exposure to Acidic and Alkaline pH†

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The molecular response of *Shewanella oneidensis* MR-1 to variations in extracellular pH was investigated based on genomewide gene expression profiling. Microarray analysis revealed that cells elicited both general and specific transcriptome responses when challenged with environmental acid (pH 4) or base (pH 10) conditions over a 60-min period. Global responses included the differential expression of genes functionally linked to amino acid metabolism, transcriptional regulation and signal transduction, transport, cell membrane structure, and oxidative stress protection. Response to acid stress included the elevated expression of genes encoding glycogen biosynthetic enzymes, phosphate transporters, and the RNA polymerase sigma-38 factor (*rpoS*), whereas the molecular response to alkaline pH was characterized by upregulation of *nhaA* and *nhaR*, which are predicted to encode an Na^+/H^+ antiporter and transcriptional activator, respectively, as well as sulfate transport and sulfur metabolism genes. Collectively, these results suggest that *S. oneidensis* modulates multiple transporters, cell envelope components, and pathways of amino acid consumption and central intermediary metabolism as part of its transcriptome response to changing external pH conditions.

Extracellular pH is an important factor influencing the cell physiology and growth of bacteria. Microorganisms commonly encounter widely fluctuating pH environments and consequently have evolved various adaptive strategies for the purpose of minimizing acid- or alkaline-induced damage (for a review, see reference 11).

Cellular mechanisms for maintaining pH homeostasis are well defined. The initial response of gram-negative bacteria, as investigated intensively in the model organism *Escherichia coli*, to acute cytoplasmic acidification is the activation of outward H^+ pumps such as K^+/proton antiporters (11, 18), while cell survival upon prolonged acid stress exposure requires further alterations in gene expression. Mechanisms responsible for acid tolerance or resistance in *E. coli* include the arginine and glutamate decarboxylase/antiporter systems, which are thought to counteract external acidification through the consumption of intracellular protons and the generation of alkaline amines (reviewed in 11). Additional acid tolerance responses involve the induction of proteins that postsynthetically modify the lipid composition of the inner membrane (8), thereby altering proton permeability.

Sodium proton antiporters, which bring in 2 H^+ for each Na^+ extruded, play a major role in the homeostatic mechanisms enabling bacteria to adapt to alkaline pH in the presence of Na^+ (30). Proteomic studies employing two-dimensional gel electrophoresis have also shown that high pH induces amino acid metabolic enzymes such as tryptophan deaminase (TnaA) and *o*-acetylserine sulfhydrylase A (CysK), which potentially enable *E. coli* to reverse alkalinization by metabolizing amino acids to produce acidic products (5, 35). In general, reports of prokaryotic tolerance and adaptation to alkaline pH have been less extensive to date compared to those of acidic pH (see reference 34 for a review of alkaline stress responses).

Shewanella oneidensis strain MR-1 (37), a facultatively anaerobic gram-negative γ-proteobacterium, is a respiratory generalist that grows well under microaerophilic conditions and is capable of anaerobic respiration using a wide variety of organic and inorganic substrates (e.g., dimethyl sulfoxide, sulfite, thiosulfite, nitrate, fumarate, Fe[III], Mn[III, and Mn[IV]) as terminal electron acceptors (22, 24–26). This metabolically versatile organism is of particular interest because of its potential utility in the in situ microbial bioreduction and immobilization of various anthropogenic pollutants, such as Cr(VI) (23, 38), and as a result, the complete DNA sequence of the *S. oneidensis* MR-1 genome has been determined (15). However, the application of such dissimilatory metal-reducing bacteria to contaminated sites can be complicated by unpredictable interactions with other microbial metabolisms and environmental

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stressors, such as variations in external pH, salinity, and oxidative conditions.

Currently, there is little information on the underlying molecular mechanisms enabling this bacterium to cope with extracellular acid and alkaline environments. In this comparative study, we examined the global transcriptional response of exponential-phase *S. oneidensis* MR-1 following exposure to both acidic (pH 4) and basic (pH 10) stress for 30 and 60 min. The DNA microarray analyses described here identified more than 600 genes that were differentially induced or repressed at least twofold under the experimental conditions tested. The extent and complexity of the *S. oneidensis* response to acidic and alkaline pH stress were reflected in the wide distribution of putative functional roles attributed to differentially expressed genes. In this study, we focused primarily on the upregulated genes, which we surmised would encode proteins most directly contributing to the pH homeostatic mechanisms of the organism, while downregulated genes could be attributed, at least in part, to cessation of cell growth under the tested conditions. This is not to say, however, that downregulated genes or genes not showing significant changes in expression based on transcriptome profiling are not important in the cell's response to pH stress.

Growth conditions and viability. *S. oneidensis* MR-1 was grown aerobically at 30°C with shaking (150 rpm) on a modified basal minimal medium containing 0.05 g/liter tryptone and 0.025 g/liter yeast extract (4) unless otherwise indicated. The modified basal minimal medium was further supplemented with 20 mM sodium lactate, 30 mM HEPES, 1 ml/liter of trace element solution, and 1 ml/liter of vitamin mix. Trace element solution was composed of 12.5 ml/liter HCl and the following (g/liter) components: 2.1 $FeSO_4$; 0.030 H_3BO_3; 0.100 $MnCl_2 \cdot 4H_2O$; 0.190 $CoCl_2 \cdot 6H_2O$; 0.024 $NiCl_2 \cdot 6H_20$; 0.002 $CuCl_2 \cdot 2H_2O$; 0.144 $Zn\ SO_4 \cdot 7H_2O$; and 0.036 Na_2MoO_4. The vitamin mixture contained these components (g/liter), which were filter sterilized and added to the modified basal minimal medium subsequent to autoclaving: 0.002 biotin, 0.002 folic acid, 0.010 pyridoxine HCl, 0.005 riboflavin, 0.005 thiamine, 0.005 nicotinic acid, 0.005 pantothenic acid, 0.0001 cyanocobalamin, 0.005 *p*-aminobenzoic acid, and 0.005 thiocitric acid. The complete medium was adjusted to pH 7.0.

For pH stress experiments, the medium was identical, substituting 30 mM 2-morpholinoethanesulfonic acid (MES) or 30 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) for HEPES and adjusting the pH to 4 and 10, respectively. Cells exposed to the different pHs were collected and serially diluted in the growth medium (pH 7.0) described above. Serial dilutions were plated on modified minimal medium supplemented with 1.5% agar to determine relative viability. Plate counts indicated no relative differences in the viability of cells exposed to pH 4 or pH 10 relative to pH 7 for both exposure times (30 and 60 min) (results not shown).

Experimental design. Three independent biological replicates of *S. oneidensis* MR-1 picked from well-isolated colonies on minimal medium agar plates were initially grown overnight (16 to 18 h) in modified minimal medium broth (described above). Each of the three flasks was subcultured into two 120-ml amounts of modified minimal medium (pH 7.0) (six flasks in total). Cultures were grown aerobically until a mid-log optical density at 600 nm of ~0.15 to 0.2 was attained. Cells from each flask in a duplicate set of independent biological replicates were split into three equal aliquots (40 ml), and the medium was replaced with an equivalent volume of fresh, warmed (at 30°C) modified minimal medium buffered with MES (pH 4), HEPES (pH 7), or CAPS (pH 10).

One set of the biological replicates was incubated aerobically at 30°C for 30 min, while the other set was allowed to incubate for 60 min. Cells were then immediately harvested by centrifugation at 4°C at maximum speed in a 5415R centrifuge (Eppendorf, Westbury, N.Y.) for 20 s, and the spent culture supernatant was removed. One of the biological replicates for the 60-min time point was irretrievably lost during this process and subsequently omitted from the experiment. Total cellular RNA was extracted using the Trizol reagent (Gibco-BRL, Carlsbad, California), treated with RNase-free DNase I (Ambion, Austin, TX), and purified using RNeasy columns (QIAGEN, Valencia, CA) according to the manufacturer's recommended protocol.

Microarray construction and hybridization experiments. Genomic microarrays for *S. oneidensis* MR-1 were fabricated as described previously (13). Fluorescently labeled cDNA copies of total cellular RNA extracted from cells exposed to acidic, basic, or neutral pH were prepared by incorporation of fluorescein-labeled nucleotide analogs during a first-strand reverse transcription reaction as described elsewhere (36). RNA from control pH 7-exposed cells was fluorescently labeled with Cy5 (or Cy3), and that from the acidic or basic pH-exposed cells was labeled with Cy3 (or Cy5). The two differentially labeled cDNA populations to be compared, neutral pH exposure (control) and acidic or basic pH exposure (treatment), were mixed and hybridized simultaneously to the array essentially as described previously (36), with the exception that 50% (vol/vol) formamide was added to the hybridization buffer. Array hybridization, washing, and scanning were performed as described elsewhere (36).

Array data analysis and validation. Analysis of global gene expression was performed using six independent array experiments (i.e., two fluorescent-dye reversal experiments in three biological replicates obtained from independently treated cultures of MR-1). In addition, each *S. oneidensis* MR-1 microarray slide contained duplicate sets of probes (i.e., two technical replicates) for each gene at different locations. This yielded a total of 12 expression measurements per gene for the 30-min pH conditions. Only two biological replicates were used for gene expression analysis for the 60-min pH conditions, resulting in a total of eight expression measurements per gene.

To determine fluorescence intensity (pixel density) and background intensity, 16-bit TIFF scanned images were analyzed using the ImaGene software package (version 5.0, Biodiscovery, Inc., Los Angeles, Calif.). Prior to channel normalization, microarray outputs were first filtered to remove spots of poor signal quality by excluding those data points with a mean intensity of <2 standard deviations above the overall background for both the Cy5 and Cy3 channels (14). Random error estimation, normalization, and false-positive correction were all accomplished using the ArrayStat software package (Imaging Research, Inc. Ontario, Canada). The pooled common error method, channel median normalization, and the false discovery rate were all applied for data analysis. The net signal of the control or treatment channel was calculated by

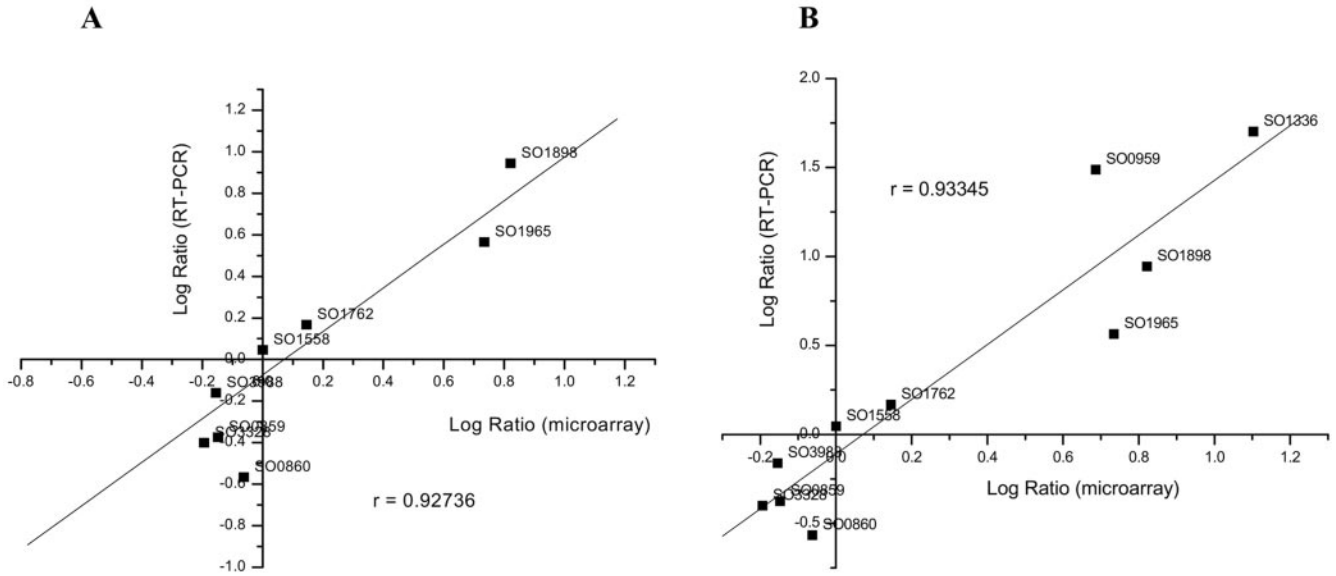

FIG. 1. Comparison of gene expression measurements by microarray hybridization and real-time quantitative RT-PCR. Genes examined included the following: *so0859* (sensory box histidine kinase/response regulator), *so0860* (response regulator), *so0959* (cytosol aminopeptidase PepA-1), *so1336* (Na^+/H^+ antiporter NhaA), *so1558* (phosphate regulon response regulator PhoB), *so1762* (transcriptional regulator, AraC/XylS family), *so1898* (transcriptional regulator, putative), *so1965* (transcriptional regulator, LysR family), *so3328* (acetyltransferase, GNAT family), and *so3988* (aerobic respiration control protein ArcA). (A) Changes (fold difference and log transformed) in gene expression in *S. oneidensis* MR-1 in response to a pH downshift from 7 to 4. (B) Changes (fold difference and log transformed) in gene expression in *S. oneidensis* MR-1 in response to a pH upshift from 7 to 10. Changes in gene expression were log transformed (in base 10), and the real-time RT-PCR $\log_{10}$ values were plotted against the microarray data $\log_{10}$ values. The correlation coefficient (r) is given.

subtracting the background (median) from the signal (median) plus 100 (a pseudo-minimum signal to enforce a positive value). If the net signal of a spot was still below zero, a value of 50 was assigned. The log ratio of the treatment and control was defined as $\log_2$ (net signal of the treatment) $-$ $\log_2$ (net signal of the control). We performed the LOWESS method (43) and the sector-based normalization of all log ratios. The final mean log ratio is averaged among all replicates, requiring $n \geq 2$. To assess the significance of the log ratio, a Z score was calculated by dividing the final mean log ratio over the square root of 0.25, a pseudovariance plus the sum of the variance among all replicates. A significant change in expression required a score of $Z \geq 1.5$ (significant induction) or $Z \leq -1.5$ (significant repression).

Real-time quantitative reverse transcription-PCR (RT-PCR) was used to provide an independent assessment of microarray expression measurements for selected genes. Real-time PCR was performed as described previously (41) using an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, Calif.). Ten genes representing a range of gene expression ratios for the 30-min acidic and basic pH-treated samples were selected for comparative real-time PCR analysis, and primer pairs were designed using the program Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) to yield a PCR product approximately 100 bp in length: *so0859* (sensory box histidine kinase/response regulator), *so0860* (response regulator), *pepA-1* (*so0959*, cytosol aminopeptidase), *nhaA* (*so1336*, Na^+/H^+ antiporter), *phoB* (*so1558*, phosphate regulon response regulator), *so1762* (AraC/Xyls family transcriptional regulator), *so1898* (putative transcriptional regulator), *so1965* (LysR family transcriptional regulator), *so3328* (GNAT family acetyltransferase), and *arcA* (*so3988*, aerobic respiration control protein). Comparison of gene expression measurements by microarray hybridization and real-time RT-PCR indicated a high level of concordance between the datasets, with Pearson correlation coefficients $r = 0.927$ and $r = 0.933$ for the 30-min pH 4 and pH 10 samples, respectively (Fig. 1).

Global gene expression patterns in response to acidic and alkaline pH. The transcriptional response of *S. oneidensis* following exposure to acidic and basic medium was pleiotropic, involving many functional classes of genes. Figure 2 provides a summary of the percentage of differentially expressed genes grouped by functional categories according to TIGR's annotation of the *S. oneidensis* MR-1 genome (15). Exposure to pH 4 for 30 min was the most potent effector of gene regulation under our experimental conditions. Of the 4,648 genes examined by microarray analysis, 1,069 genes (23% of the total number of open reading frames represented on the array) were identified as being significantly up- or downregulated ($P < 0.05$, absolute Z score ≥ 1.5) 30 min after exposure to acidic conditions, while alkaline pH challenge resulted in a total of 662 genes (14%) being differentially expressed at the same time interval (Table 1). More genes were repressed than were induced in response to low and high external pH. For example, 384 (~8%) of the arrayed genes were overexpressed, while 685 (~15%) were underexpressed following 30 min at pH 4 (Table 1). Prolonged exposure to pH 4 resulted in an overall decrease in the number of differentially expressed genes, with only 7% being induced and 9% repressed at the 60-min time interval. Interestingly, this was in contrast to the transcriptome profile generated in response to 60-min exposure to pH 10, which

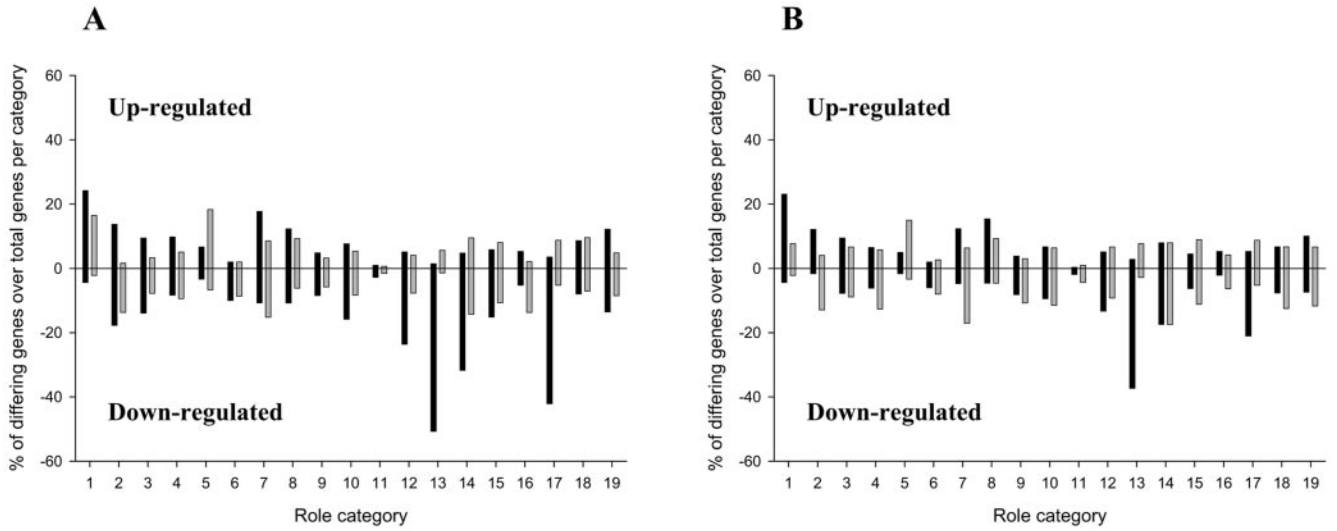

FIG. 2. Number of differentially expressed genes represented as a percentage of the total genes per functional classification according to the Institute for Genomic Research *S. oneidensis* genome database. The functional classes of genes affected by external pH 4 (solid bars) and pH 10 (shaded bars) are compared for the 30-min (A) and 60-min (B) exposure intervals. Columns: 1, amino acid biosynthesis; 2, cofactors and prosthetic groups; 3, cell envelope; 4, cellular processes; 5, central intermediary metabolism; 6, DNA metabolism; 7, energy metabolism; 8, fatty acid metabolism; 9, hypothetical proteins; 10, conserved hypothetical proteins; 11, other categories; 12, protein fate; 13, protein synthesis; 14, purines and pyrimidines; 15, regulatory functions; 16, signal transduction; 17, transcription; 18, transport and binding protein; and 19, unknown function. Columns above 0 represent the percentage of genes whose expression increased in response to pH 4 or pH 10; columns below 0 represent the percentage of genes whose expression decreased in response to pH 4 or pH 10.

resulted in a greater number of differentially expressed genes compared to the 30-min exposure (Table 1).

While there were notable distinctions in the response of MR-1 to an acidic or alkaline environment (described in more detail below), comparison of the global gene expression profiles obtained under the different pH conditions for the 30-min time interval indicated that the following functional classes were similarly affected in terms of the relative percentage levels of induced and repressed genes: amino acid biosynthesis (17 to 24% upregulated versus 2 to 4% downregulated), DNA metabolism (2% up versus 9 to 10% down), hypothetical (3 to 5% up versus 6 to 8% down), regulatory functions (6 to 8% up versus 11 to 15% down), transport and binding proteins (9 to 10% up versus 7 to 8% down), and other categories (0.6 to 0.9% up versus 2 to 3% down) (Fig. 2A).

TABLE 1. Number of genes induced or repressed at least twofold in transcriptome profiling experiments at different time intervals following acidic pH and basic pH exposure

External pH	Time (min) after down- or upshift from pH 7	No. of genes:		Total no. of genes
		Induced	Repressed	
4	30	384	685	1,069
	60	327	420	747
	Unique[a]	ND[b]	ND	115
10	30	265	397	662
	60	279	530	809
	Unique	ND	ND	53
4 and 10	Common	ND	ND	73

[a] Total number of genes that were differentially expressed only under acid or basic conditions at the 30- and 60-min time points.
[b] ND, not determined.

Seventy-three genes exhibited differential expression under both low and high extracellular pH (Table 1). This group of common pH-dependent responders included genes encoding two GNAT family acetyltransferases (*so0877* and *so1049*), which play important roles in the production of antioxidant thiols such as glutathione (10), and a hypothetical gene (*so3762*) that was induced under all pH conditions. The repression of the catabolite activator gene *crp*, a global regulator that positively or negatively controls a vast number of genes involved in various functions in *Escherichia coli* (6), as well as genes encoding the RNA polymerase sigma-70 factor (*so3096*), the potassium uptake protein TrkH (*so0028*), a putative membrane protein (*so1048*), and a number of poorly characterized proteins (e.g., *so1053*, *so3047*, *so3719*, *so3720*, *so3900*, *so4126*, *so4355*, *so4362*, and *so4621*) constituted part of a general transcriptome response to changes in external pH. Other differentially expressed genes showed either induction under pH 4 and repression under pH 10 (e.g., *so1753*, *so1813*, and *so3373*) or repression under pH 4 and induction under pH 10 (e.g., *so0551*, *so0552*, *so0753*, *so0808*, *so1869*, *so2632*, *so3315*, and *so4073*). For the most part, these genes encoded hypothetical or conserved hypothetical proteins, with the exception of *so1813*, which encodes a putative DNA-binding protein.

The gene expression trends in *S. oneidensis* MR-1 in response to acid stress showed some notable distinctions compared to external alkaline pH exposure. A greater percentage of genes, for example, were upregulated in the functional categories of cofactors and prosthetic groups, cell envelope proteins, and energy metabolism in response to pH 4 compared to

TABLE 2. Selected *S. oneidensis* genes upregulated ≥2-fold at one or more time points in response to external acidic pH

Gene	Gene product description	Induction ratio[a] (pH 4/pH 7)	
		30 min	60 min
so0020	Fatty oxidation complex, beta subunit (*fadA*)	5.3	2.2
so0021	Fatty oxidation complex, alpha subunit (*fadB*)	3.8	2.7
so0184	Conserved hypothetical protein	18.4	11.2
so0185	Conserved hypothetical protein	12.8	7.2
so0576	PhoH family protein	12.7	4.5
so0818	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (*metE*)	15.2	NA
so0859	Sensory box histidine kinase/response regulator	5.5	3.9
so0860	Response regulator	2.1	NA
so0866	Minor curlin subunit CsgB, putative	21.7	12.6
so1239	Conserved hypothetical protein	19.5	6.1
so1307	Aquaporin Z (*aqpZ*)	3.3	2.1
so1324	Glutamate synthase, small subunit (*gltD*)	3.5	2.2
so1325	Glutamate synthase, large subunit (*gltB*)	3.9	3.0
so1494	1,4-α-Glucan branching enzyme (*glgB*)	41.6	3.3
so1495	Glycogen operon protein (*glgX*)	21.6	2.6
so1496	Glycogen phosphorylase family protein	20.5	2.5
so1498	Glucose-1-phosphate adenylyltransferase (*glgC*)	9.7	1.8
so1499	Glycogen synthase (*glgA*)	9.3	2.2
so1557	Outer membrane porin, putative	9.6	3.9
so1558	Phosphate regulon response regulator PhoB (*phoB*)	4.8	2.6
so1724	Phosphate ABC transporter, permease protein, putative	4.0	2.7
so1725	Phosphate ABC transporter, ATP-binding protein (*pstB-1*)	7.8	4.7
so1726	Phosphate transport system regulatory protein PhoU (*phoU*)	5.8	3.3
so1762	Transcriptional regulator, AraC/XylS family	10.3	5.8
so1813	DNA-binding protein, putative	12.1	2.8
so1844	Extracellular nuclease, putative	13.5	4.1
so1975	Zinc carboxypeptidase-related protein	11.3	7.6
so2001	5′-Nucleotidase (*ushA*)	12.7	6.3
so2002	Hypothetical protein	17.9	7.4
so2101	Lipoprotein, putative	5.5	NA
so2385	Conserved hypothetical protein	22.2	4.0
so2519	Transcriptional regulator, AraC family	11.9	4.5
so2570	Lipoprotein, putative	5.7	3.3
so3432	RNA polymerase sigma-38 factor (*rpoS*)	4.3	3.0
so3433	Lipoprotein NlpD (*nlpD*)	3.2	2.6
so3687	Curli production assembly/transport component CsgE, putative	11.0	11.1
so4195	PAP2 family protein	15.7	7.8
so4405	Catalase/peroxidase hydroperoxidase (*katG-2*)	14.8	3.6
so4466	Methyl-accepting chemotaxis protein	10.7	5.1

[a] Relative gene expression is presented as the mean ratio of the fluorescence intensity of pH 4-exposed cells to that of pH 7-exposed control cells for time periods of 30 and 60 min. NA, not available.

pH 10. More than 40% of the genes assigned a function in protein synthesis (51%) and transcription (42%) were downregulated in response to pH 4 at the 30-min exposure interval (Fig. 2A, bars 13 and 17), indicating that these primary cellular processes are largely negatively impacted by acid stress. The global transcriptional response to high extracellular pH, by contrast, was distinguished by a high percentage of upregulated genes (18 and 15% at 30 and 60 min, respectively) with predicted functions in central intermediary metabolism (Fig. 2A, bar 5).

Substantially fewer genes with functional roles in signal transduction were affected at the 60-min time point for pH 10 exposure than the 30-min time point (Fig. 2A and B, bar 16).

Certain acid-induced stress genes have been shown to be coinduced by conditions such as oxidative stress, e.g., the alkyl hydroperoxide reductase *ahpC* (4, 20). Genes encoding products known to function in oxidative stress protection also showed differential expression in *S. oneidensis* under both acidic and basic conditions. Expression of the catalase/peroxidase gene, *katG-2*, was regulated by low pH, exhibiting induction of 15- and 4-fold at the 30- and 60-min time points, respectively (Table 2). By contrast, the Fe-dependent superoxide dismutase gene *sodB* and *ahpC* showed base-induced expression (Table 2 and Table S1 in the supplemental material). While oxidative stress genes were upregulated under both treatments, uniquely expressed genes under the different growth conditions could be construed as pH responsive (Table 1).

Genomic response of *S. oneidensis* to acid stress. Microarray analysis of *S. oneidensis* cells exposed to an acid environment revealed multiple effects on gene expression, resulting in a rapid and broad adjustment of its transcriptional program. This molecular response was characterized predominantly by overexpression of genes with predicted functions in amino acid biosynthesis, cell envelope and membrane structure, transcriptional regulation and signal transduction, phosphate transport, and chemotaxis. A subset of genes induced under acid stress conditions is provided in Table 2 (see Table S1 in the supplemental material for the complete microarray data set). We

focus on the major features of the MR-1 transcriptome response to acid stress below.

Induction of cell envelope components following acidic pH exposure. As an environmental organism capable of metal reduction, much of *S. oneidensis* function occurs in the outer membrane and periplasm, compartments most readily impacted by extracellular pH. Thus, not surprisingly, a number of cell membrane components showed pH-dependent expression in our study. Approximately 9% of the total number of genes assigned to the functional category of cell envelope exhibited upregulated expression under acid stress (30-min time point), while 14% of such annotated genes showed repression (Fig. 2A). Genes encoding putative lipoproteins (*so2101*, *so2570*, and *nlpD*), outer membrane proteins (*so1781*, *so2375*, *so3708*, and *so4321*), efflux proteins (*so4090*), and porins (*aqpZ*, *mscL*, and *so1557*) displayed a 3- to 10-fold range in induction at the 30-min time point and for the most part continued to show upregulated expression (albeit at lower levels) 60 min following exposure to pH 4. This suggests that *S. oneidensis* might minimize acid-induced damage by altering proton permeability via modifications in the composition and structure of the cell membrane.

Other upregulated genes with functions related to cell surface structures included ORFs for curli biogenesis. Curli are highly adhesive proteinaceous extracellular fibers that have been shown to promote both initial adhesion and cell-cell interaction during biofilm development in *E. coli* and *Salmonella enteritidis* (1, 32, 39). Based on the annotation of the *S. oneidensis* genome, the putative genes encoding curli assembly subunits are *csgB* (*so0866*) and the *csgEFG* operon (*so3687 to so85*) (15). *csgB* was increased in expression approximately 22-fold in response to environmental acidification (30-min time point), and the three genes constituting the *csgEFG* operon were upregulated 11-, 3.2-, and 4.1-fold, respectively (see Table S1 in the supplemental material). These genes continued to display induction after 60 min and only in response to acid stress.

Induction of genes with regulatory or signal transduction functions in response to acid stress. A number of regulatory and signal transduction genes appear to play roles in the response of *S. oneidensis* to an acid environment. Two functionally undefined transcriptional regulatory genes, *so1762* (encoding a transcriptional regulator of the AraC/Xyls family) and *so2519* (encoding an AraC family regulator), were induced at the highest levels, 10- and 12-fold, respectively, under 30-min acid exposure compared to other regulatory genes. The upregulation of *so1762* was confirmed by real-time PCR (7.12- ± 2.06-fold) (Fig. 1A; see Table S2 in the supplemental material). Following acid exposure for 60 min, the expression of *so1762* and *so2519* was still induced, albeit at reduced magnitudes (four- to sixfold). Similarly, gene *so0859*, encoding a sensory box histidine kinase/response regulator of a functionally uncharacterized two-component signal transduction system, was upregulated 30 min after acid treatment, and the microarray expression measurements for this gene correlated highly with real-time PCR measurements (5.46-fold compared to 5.54- ± 1.38-fold, respectively; see Table S2 in the supplemental material). Gene *so0860* (induced ~2-fold after a 30-min exposure), which encodes a response regulator, is located immediately downstream of *so0859* and is transcribed in the same direction. The proximity and coregulated expression pattern of *so0860* suggest that the gene products of *so0859* and *so0860* comprise a two-component regulatory system involved in the acid stress response. Other functionally undefined transcriptional regulatory genes of the LuxR family (*so0864* and *so2725*) were also upregulated.

In other organisms such as the enteric bacterium *Salmonella enterica* serovar Typhimurium, low external pH alters the expression of several regulatory genes, including the RNA polymerase σ^s factor encoded by *rpoS*, the iron regulatory factor encoded by *fur*, and the two-component signal transduction system encoded by *phoPQ* (12). RpoS is a global regulator that also controls numerous genes expressed in response to starvation and entry into the stationary growth phase (16). Transcriptome profiling revealed that transcription of *S. oneidensis rpoS* (*so3432*) increased approximately four- and threefold in response to external acid conditions (30-min and 60-min time points, respectively), while the expression of *fur* (*so1937*) was not significantly changed. By contrast, expression of *phoP* (*so1946*) and *phoQ* (*so1945*) was actually repressed about threefold under acid stress conditions (30-min interval).

It has been shown that the σ^s-dependent acid tolerance response is required for *Salmonella enterica* to survive acid stress imposed by volatile fatty acids and contributes to inorganic acid tolerance (2). However, the PhoPQ regulatory system was found to be nonessential for log-phase acid tolerance in *Salmonella* in the presence of a functional σ^s (3). Based on the array results of this study, preferential upregulation of *S. oneidensis rpoS* under acid stress conditions, and not alkaline pH exposure, suggests a regulatory role for this alternative sigma factor in controlling the expression of genes contributing to the acid survival mechanisms of *S. oneidensis*.

Upregulation of metabolic genes in response to acid stress. Genes that theoretically could contribute to alkalization of the cytoplasm are referred to as pH homeostatic (42). One type of pH-homeostatic system that has been extensively described in *E. coli* is the amino acid decarboxylases, particularly arginine and glutamate decarboxylases, which are thought to provide protection against acid stress through the consumption of intracellular protons (for a review, see reference 11). In *E. coli*, the two genes encoding glutamate decarboxylase (*gadA* and *gadB*) are induced during entry into stationary phase or by acidic pH in log phase during growth in minimal medium (7, 44). In this study, about 24% of the total number of *S. oneidensis* genes assigned an amino acid biosynthesis function were upregulated at both time points in response to pH 4, while only 4% of the genes in this functional category were downregulated under the same conditions. However, genes encoding a putative glutamate decarboxylase (*so1769*) and biosynthetic arginine decarboxylase (*so1870*) in *S. oneidensis* were slightly repressed (1.5- to 1.7-fold) under low pH in supplemented minimal medium. On the other hand, *gltB* and *gltD*, which encode the subunits of glutamate synthase, were induced 3.9- and 3.5-fold, respectively, in response to a 30-min exposure to pH 4 and showed only slightly lower upregulated levels at the 60-min time point.

Genes with annotated functions in fatty acid β-oxidation (*fadBA*) and glycogen biosynthesis (*glgB*, *glgX*, *so1496*, *glgC*, and *glgA*) also showed significant (>2-fold) increases in mRNA abundance (Table 2) and presumably contribute to the

organism's ability to survive acid stress during log-phase growth in minimal medium. In *E. coli*, the *fadBA* operon encodes a multifunctional enzyme complex that exhibits enoyl-coenzyme A (CoA) hydratase (EC 4.2.1.17), 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), *cis*-Δ^3-*trans*-Δ^2-enoyl-CoA isomerase (EC 5.3.3.3), 3-hydroxyacyl-CoA epimerase, and 3-ketoacyl-CoA thiolase (EC 2.3.1.16) activities (31). The *E. coli fadR* gene product functions as a repressor of the fatty acid degradative (*fad*) operon (9; reviewed in reference 27). The *S. oneidensis fadR* homolog (*so2885*) is downregulated approximately twofold in response to acidic pH, which is consistent with its functional role as a negative regulator of *fadAB* expression. Inducible fatty acid-oxidizing systems might aid in the acid survival of *S. oneidensis* by effecting changes in membrane fatty acid composition which, in turn, alter proton permeability. However, it is not clear how or whether increased expression of genes involved in glycogen biosynthesis contributes to the ability of *S. oneidensis* MR-1 to cope with acid stress conditions.

Repression of protein synthesis and universal stress genes after acidic pH exposure. A preponderance of underexpressed genes characterized the molecular response of *S. oneidensis* to acid stress. For example, numerous genes exhibiting a downregulated coexpression pattern were found to encode ribosomal proteins (including *so0219-so0257* and *so3927* to *so3940*). The repression of these genes indicated an arrest of overall protein synthesis presumably due to growth cessation, while cells apparently redirected energy toward the increased expression of genes more directly involved in the protective response to an external acid environment. In addition, various genes (*dnaK*, *dnaJ*, *grpE*, *groES-groEL*, *hslVU*, *htpG*, *lon*, and *clpB*) encoding homologs of chaperones, chaperonins, classical heat shock proteins, and stress-related proteases were downregulated, in most cases strongly (e.g., 7- to 17-fold at the 30-min time point), at the transcription level in response to acidic pH for the time intervals examined. This suggests that DnaK, GroE, and other chaperone machines do not play a central role in acid tolerance or the regulation of adaptation to acidic conditions in *S. oneidensis*. The lack of induction of chaperone or universal stress genes is contrary to what has been observed in *S. oneidensis* during heat shock (13) or in other bacteria during acid stress (19, 21). Changes in the levels of DnaK and GroE profoundly influenced the tolerance of *Streptococcus mutans* to environmental acidification (19).

Genomic response of *S. oneidensis* to alkaline pH. Exposure of *S. oneidensis* to extracellular alkaline pH stress resulted in a total of 662 genes (~14% of the total MR-1 genes represented on the array) and 809 genes (~17%) showing significant twofold or greater changes ($P < 0.05$, absolute Z score ≥ 1.5) in expression at the 30- and 60-min time points, respectively. In general, amino acid biosynthesis and central intermediary metabolism were affected initially the most, with 17 and 18%, respectively, of the total number of genes assigned to these functional categories showing ≥2-fold induction under pH 10 exposure (30 min) (see Table 3 for a subset of induced genes and Table S1 in the supplemental material for the complete microarray data set). With longer exposure to alkaline pH (i.e., the 60-min time point), the percentage of upregulated amino acid biosynthetic genes decreased to 8%.

Induction of genes involved in sulfate transport and assimilative sulfur metabolism following exposure to basic pH. One of the major features of the *S. oneidensis* response to extracellular alkaline pH was the upregulation of central intermediary metabolic genes with specific functions in assimilative sulfur metabolism. Inorganic sulfate is reduced and incorporated into bioorganic compounds via a pathway termed assimilatory sulfate reduction, which is the major route of cysteine biosynthesis in most microorganisms (reviewed in 17). Genes encoding the enzymes adenylylsulfate kinase (*cysC*), sulfate adenylyltransferase (*cysDN*), phosphoadenosine phosphosulfate reductase (*cysH*), and sulfite reductase (*cysIJ*) were highly induced (up to 36-fold) at the 30-min time point, as were two sulfate ABC transport systems (Table 3).

cysND, *cysC*, *cysH*, and *cysIJ* encode enzymes responsible for the activation and subsequent reduction of sulfate to sulfide. An *S. oneidensis* homolog of cysteine synthase (CysK), which synthesizes cysteine from *O*-acetylserine and sulfide, also displayed increased transcription in response to an alkaline environment (Table 3). Expression of *cysK* has been reported to be a general bacterial response to such stresses as tellurite, hydrogen peroxide, acid, and diamide (20, 40). The induction of *S. oneidensis* genes encoding cysteine synthase (*cysK*), glutathione reductase (*so4782*), and superoxide dismutase (*sodB* and *so2881*) is perhaps a general protective response of MR-1 cells to oxidative stress generated by extracellular alkalinity. High pH appears to enhance the cellular demand for cysteine, which may be needed to repair oxidatively damaged iron-sulfur cluster proteins with crucial roles in metabolism, although experimental evidence is needed to substantiate this.

Induction of a putative Na^+/H^+ antiporter in response to alkaline pH. The transcriptomic response of *S. oneidensis* to alkaline pH was distinguished by the induction (greater than tenfold) of *nhaA* (*so1336*), which encodes a Na^+/H^+ antiporter, and *nhaR* (*so1338*), which encodes a transcriptional activator protein (Table 3). Upregulation of *nhaA* was confirmed by real-time PCR (Fig. 1B). Open reading frame *so1337*, which encodes a hypothetical protein (128 amino acids in size), is positioned immediately downstream of *nhaA* and may form a single transcriptional unit with *nhaA* and *nhaR*, although this remains to be confirmed. The functionally undefined gene shows the highest induction at both the 30-min (97-fold) and 60-min (40-fold) time points under alkaline stress conditions (Table 3).

Based on its proximity to *nhaA* and *nhaR* and coregulated expression in an alkaline pH context, *so1337* might function as part of this putative sodium proton antiporter system. The membrane-bound Na^+/H^+ antiporter encoded by *nhaA* is the main *E. coli* system responsible for adaptation to Na^+ and alkaline pH (in the presence of Na^+) (28). Based on the transcriptome data presented here, the *S. oneidensis nhaA* gene product likely plays a major role in the cellular response to alkaline pH. The presence of a *S. oneidensis* homolog of NhaR, which has been shown to positively control *nhaA* expression in *E. coli* (33), suggests that the regulatory mechanism is conserved in MR-1. *S. oneidensis* genes *nhaB* (*so2886*) and *nhaD* (*so0935*), encoding two other predicted sodium proton antiporter systems, were both repressed approximately threefold in response to a 30-min exposure to external pH 10, while *nhaB* was upregulated about twofold at the 60-min time point only

TABLE 3. Selected *S. oneidensis* genes upregulated ≥2-fold at one or more time points in response to external alkaline pH

Gene	Gene product description	Induction ratio[a] (pH 10/pH 7)	
		30 min	60 min
so1336	Na^+/H^+ antiporter (*nhaA*)	12.7	12.6
so1337	Hypothetical protein	97.0	39.8
so1338	Transcriptional activator protein NhaR (*nhaR*)	17.7	10.6
so1339	Conserved hypothetical protein	6.3	3.9
so1417	Sensor histidine kinase	6.7	1.8
so1689	Cation transport ATPase, E1-E2 family	10.3	2.8
so1870	Biosynthetic arginine decarboxylase (*speA*)	3.7	1.8
so1896	3-Methylcrotonyl-CoA carboxylase, beta subunit (*pccB-1*)	7.9	8.9
so1897	Isovaleryl-CoA dehydrogenase (*ivd*)	7.9	9.7
so1898	Transcriptional regulator, putative	6.6	6.7
so1946	Transcriptional regulatory protein PhoP (*phoP*)	3.2	2.6
so1965	Transcriptional regulator, LysR family	5.4	4.5
so2881	Superoxide dismutase (*sodB*)	2.7	3.3
so2903	Cysteine synthase A (*cysK*)	5.8	2.7
so3142	Peptidyl-dipeptidase Dcp (*dcp-1*)	7.4	5.5
so3599	Sulfate ABC transporter, periplasmic sulfate-binding protein (*cysP*)	21.7	4.7
so3600	Sulfate ABC transporter, permease protein (*cysT-1*)	7.4	1.6
so3601	Sulfate ABC transporter, permease protein (*cysW-1*)	7.2	1.9
so3602	Sulfate ABC transporter, ATP-binding protein (*cysA-1*)	14.7	2.0
so3723	Adenylylsulfate kinase (*cysC*)	5.7	1.9
so3726	Sulfate adenylyltransferase, subunit 1 (*cysN*)	20.5	2.6
so3727	Sulfate adenylyltransferase, subunit 2 (*cysD*)	30.2	3.5
so3728	Uroporphyrin-III C-methyltransferase (*cobA*)	35.9	5.5
so3736	Phosphoadenosine phosphosulfate reductase (*cysH*)	6.7	1.5
so3737	Sulfite reductase (NADPH) hemoprotein beta-component (*cysI*)	9.8	1.8
so3738	Sulfite reductase (NADPH) flavoprotein alpha-component (*cysJ*)	13.9	2.8
so3739	Hypothetical protein	7.5	1.6
so3758	Conserved hypothetical protein	20.8	4.6
so3764	Conserved hypothetical protein	13.1	4.3
so3765	Conserved hypothetical protein	16.0	12.1
so3766	Hypothetical protein	15.2	11.7
so4652	Sulfate ABC transporter, periplasmic sulfate-binding protein (*sbp*)	36.8	6.1
so4654	Sulfate ABC transporter, permease protein (*cysW-2*)	8.1	1.8
so4655	Sulfate ABC transporter, ATP-binding protein (*cysA-2*)	6.7	1.6

[a] Relative gene expression is presented as the mean ratio of the fluorescence intensity of pH 4-exposed cells to that of pH 7-exposed control cells for time periods of 30 and 60 min.

under acid stress. Whereas NhaA activity has been shown to be highly dependent on pH (increasing upon alkalinization), the *E. coli* NhaB system is totally independent of pH (29).

Regulatory and signal transduction genes induced upon alkaline pH stress. A number of functionally undefined regulatory genes were induced only under alkaline conditions (Table 3). The most highly upregulated genes assigned a regulatory or signal transduction function included the following: *so1417*, encoding a sensor histidine kinase (induced 6.7-fold at the 30-min time point only); *so1898*, a putative transcriptional regulator (induced ~7-fold at both time points by microarray analysis and 8.8- ± 1.18-fold by real-time RT-PCR); *so1965*, encoding a LysR family regulatory protein (induced ~5-fold at both time points by microarray analysis and 3.7-fold ± 0.595-fold by real-time RT-PCR); and *so0443*, encoding a MerR family transcriptional regulator (induced 4.5-fold at the 30-min time point).

The genes *pccB-1* (*so1896*) and *ivd* (*so1897*), which are both induced eightfold in response to alkaline pH (Table 2), are located immediately downstream of the putative transcriptional regulatory gene *so1898*, suggesting that the *so1898* gene product might regulate the expression of these genes belonging to the functional category of energy metabolism (specifically, amino acids and amines). In contrast to acid stress, *rpoS* was slightly repressed (1.8-fold) at the 30-min interval under alkaline pH conditions, while genes encoding the transcriptional regulatory protein PhoP and its cognate sensor protein PhoQ were both upregulated slightly (2.6- and 1.8-fold, respectively) 60 min after alkaline pH challenge (see Table S1 in the supplemental material).

Summary. The present study provides an initial, comprehensive description of the global transcriptional responses elicited by *S. oneidensis* MR-1 cells when challenged with acid and alkaline pH stress. Whole-genome microarray analysis revealed that acid and alkaline environments had multiple effects on the *S. oneidensis* transcriptome, with exposure to pH 4 (30-min time interval) constituting the most potent effector of gene regulation under the experimental conditions used. In general, diverse transcriptional response patterns were observed, suggesting that the molecular effects of external pH are multifarious and likely intersect with other environmental factors such as cell growth phase, medium composition, and metabolites to elicit transcriptional responses that are both directly and indirectly related to acid and alkaline pH stress.

The response to acid stress was characterized, most notably, by the induction of genes with annotated functions in cell envelope structure (e.g., *csg* genes and *so1557*), glycogen biosynthesis (putative *glg* operon), fatty acid metabolism (*fadBA*),

glutamate synthesis (*gltBD*), phosphate transport (*so1724* and *pstB-1*), and regulation (e.g., *rpoS* and *phoU*). pH-responsive regulatory mechanisms potentially involved the alternative sigma-38 factor encoded by *rpoS*, which was upregulated at the transcript level specifically in response to low-pH exposure, and a potential two-component signal transduction system (*so0859* and *so0860*) that might be involved in the response of MR-1 to an acidifying environment.

By contrast, *S. oneidensis* appears to modulate its transcriptome in response to an alkaline environment primarily by affecting the expression of genes involved in central intermediary metabolism (particularly assimilative sulfur metabolism), sulfate transport, and Na^+/H^+ antiporter systems. The array data suggest that, similar to *E. coli*, *S. oneidensis* utilizes an Na^+/H^+ antiporter encoded by *nhaA* (*so1336*) as a strategy for coping with alkaline pH in the presence of Na^+, and *nhaA* expression may be regulated by NhaR, although experimental verification is needed. The putative NhaA antiporter, along with a hypothetical protein encoded by gene *so1337*, may function in a pH-homeostatic mechanism enabling *S. oneidensis* to survive and adapt to external alkaline conditions.

Overall, this microarray study identifies sets of putative coregulated genes and enables the generation of testable hypotheses of possible functions for their encoded products. Such global analyses provide data necessary for future examination of pH-linked transcriptional networks in *S. oneidensis* MR-1.

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