

Global profiling of *Shewanella oneidensis* MR-1: Expression of hypothetical genes and improved functional annotations

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The γ -proteobacterium *Shewanella oneidensis* strain MR-1 is a metabolically versatile organism that can reduce a wide range of organic compounds, metal ions, and radionuclides. Similar to most other sequenced organisms, $\approx 40\%$ of the predicted ORFs in the *S. oneidensis* genome were annotated as uncharacterized “hypothetical” genes. We implemented an integrative approach by using experimental and computational analyses to provide more detailed insight into gene function. Global expression profiles were determined for cells after UV irradiation and under aerobic and suboxic growth conditions. Transcriptomic and proteomic analyses confidently identified 538 hypothetical genes as expressed in *S. oneidensis* cells both as mRNAs and proteins (33% of all predicted hypothetical proteins). Publicly available analysis tools and databases and the expression data were applied to improve the annotation of these genes. The annotation results were scored by using a seven-category schema that ranked both confidence and precision of the functional assignment. We were able to identify homologs for nearly all of these hypothetical proteins (97%), but could confidently assign exact biochemical functions for only 16 proteins (category 1; 3%). Altogether, computational and experimental evidence provided functional assignments or insights for 240 more genes (categories 2–5; 45%). These functional annotations advance our understanding of genes involved in vital cellular processes, including energy conversion, ion transport, secondary metabolism, and signal transduction. We propose that this integrative approach offers a valuable means to undertake the enormous challenge of characterizing the rapidly growing number of hypothetical proteins with each newly sequenced genome.

computational biology | expression analysis | microarrays | proteomics | integrative microbiology

S*hewanella oneidensis* strain MR-1 is a facultatively anaerobic γ -proteobacterium that can use a broad range of electron acceptors for anaerobic respiration, including organic compounds, metal ions, and radionuclides (1). It is currently the subject of comprehensive study by the *Shewanella* Federation, a multiinstitutional consortium supported through the U.S. Department of Energy *Genomics: GTL* program (ref. 2; <http://doegenomestolive.org>). More than 2 years ago the *S. oneidensis* genome was sequenced and thoroughly annotated (3) with 4,931 predicted ORFs, 1,988 of which were considered uncharacterized “hypothetical” (40%). Since then, several publications have addressed issues regarding the *S. oneidensis* genome, including a correction of the total number of

the predicted genes and analysis of genes designated as hypothetical (4–6). Our current estimate includes 4,467 predicted genes, 1,623 of which are annotated as hypothetical (36%).

Although this result represents an improvement, it also serves to point out one of the emerging challenges of modern biology: namely, the rapid accumulation of uncharacterized hypothetical genes (7–11). This assignment is given to genes that have not been experimentally characterized and whose functions cannot be deduced from simple sequence comparisons. Although analytical approaches are now available for comprehensive measurements of gene and protein expression, the lack of knowledge of the function of a large proportion of this genome limits our ability to take full advantage of capabilities for advancing biology to a more predictive science. Even the prediction that these genes encode proteins, that these proteins are intact (e.g., not truncated by errors in the genome sequence), and that they are expressed in living cells is uncertain. Nonetheless, every new sequencing project results in hundreds or even thousands of new hypothetical genes. For example, the recent sequencing of Sargasso Sea microbial communities resulted in a large number of uncharacterized genes ($\approx 69,900$) grouped into $\approx 15,600$ families (12). Experimental characterizations of new proteins from one of the most extensively studied organisms, *Escherichia coli* strain K-12, are producing 20 to 30 new functional characterizations per year (13). At this rate, more than half of a century will be required to determine the biological functions of all *S. oneidensis* hypothetical genes. There is an obvious need for new approaches for rapid functional characterization of these hypothetical genes.

To address this problem, we have begun a four-phase program: first, to experimentally evaluate the expression of hypothetical genes under various conditions; second, to validate that these genes encode expressed proteins; third, to propose, by using various approaches, the most likely function of the expressed proteins; and fourth, to experimentally verify these functions. This study describes the first three phases of this program. The first and second phases herein involved a comprehensive experimental dataset that includes both microarray and proteomics expression data from multiple experiments. These analyses confidently identified 538

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Abbreviations: COG, Clusters of Orthologous Groups; LC, liquid chromatography.

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Table 1. Seven-category schema for annotating proteins

Category no.	Description	No. of genes (% of 538)
1	Exact biochemical function, based on high similarity to experimentally characterized closely related homolog	16 (3)
2	Well defined biochemical function, unknown specificity	16 (3)
3	General biochemical function, based on family/superfamily assignment and/or a conserved sequence motif	66 (12)
4	General biological function derived from the domain organization, genome context (e.g., operons), experimental (e.g., protein-protein interactions), and/or structural genomics data (e.g., similarities to proteins with known 3D structures)	86 (16)
5	Certain functional insights derived from the above data	72 (13)
6	Widely conserved protein, expressed under certain growth condition(s); e.g., "Conserved expressed protein"	190 (35)
7	Organism- or genus-specific protein, expressed under certain growth condition(s); e.g., "Expressed protein in <i>Shewanella</i> "	94 (17)

their confidence and precision. The seven-category general schema introduced here (Table 1) attempts to address these issues, classifying functional assignments based on the degree of sequence similarity to the experimentally characterized homologs and the availability of supporting data. The first category includes genes that have high levels of sequence identity to well characterized proteins from other species and can be confidently predicted to have the same function (Table 2). The second category (Table 3) includes proteins with lower, but significant, levels of sequence similarity to experimentally characterized homologs, so that their biochemical function can be well defined, although the substrate (or ligand) specificity remains unclear. The third category includes proteins that share only low-level sequence similarity (typically limited to the common active site motifs) to experimentally studied homologs and can be given only a general biochemical functional assignment (Table 5, which is published as supporting information on the PNAS web site), common for all of the proteins of a given (super)family. The fourth category contains genes whose products cannot be assigned a biochemical function but participate in a known biological process, such as cell division, membrane transport (Table 6, which is published as supporting information on the PNAS web site). The fifth category consists of uncharacterized proteins for

which only certain functional insights can be obtained (Table 7, which is published as supporting information on the PNAS web site). The sixth category consists of widely conserved expressed proteins (Table 8, which is published as supporting information on the PNAS web site). Finally, the seventh category includes all of the remaining organism- or genus-specific (herein *Shewanella*-specific) expressed proteins (Table 9, which is published as supporting information on the PNAS web site).

Exact Biochemical Functions. The original genome sequence analysis of *S. oneidensis* (3) resulted in the annotation of all 538 genes of interest as hypothetical, but one would expect that, with time, additional evidence for the function of some of these genes would become available. Indeed, searches of public databases (29–32, 34) revealed some proteins that can now be functionally annotated based on the experimental characterization of close homologs in other organisms. Unfortunately, this set represents only 16 of these 538 proteins that have been assigned exact biochemical functions [Tables 1 (category 1) and 2]. In a >2-year period of experimental efforts by the entire community, the exact biochemical character-

Table 2. Annotations of proteins with exact biochemical function: Category 1

SO ID no.	Upgraded annotation	COG no.	Pfam no.
SO0332	Homoserine kinase, type II	2334	06111
SO0342	PrpF protein required for repair/synthesis of Fe-S center of AcnD	2828	04303
SO0506	3-octaprenyl-4-hydroxybenzoate decarboxylase UbiD	0043	01977
SO0887	Peptidylarginine deiminase	2957	04371
SO1523	NAD kinase	0061	01513
SO1597	ω -3 polyunsaturated fatty acid synthase PfaD subunit	2070	03060
SO1789	UDP-2,3-diacetylglucosamine hydrolase	2908	00149
SO1963	Homogenetisate 1,2-dioxygenase	3508	04209
SO2593	NAD-specific glutamate dehydrogenase	2902	05088
SO2614	Aminodeoxychorismate lyase	1559	02618
SO2627	ATP-dependent Clp protease adaptor protein ClpS	2127	02617
SO3340	Mechanosensitive ion channel protein MscS	0668	00924
SO3436	tRNA pseudouridine synthase TruD	0585	01142
SO4413	Kynureninase	0520	00266
SO4680	CDP-glycerol:poly(glycerophosphate) glycerophosphotransferase	1887	04464
SO4719	Periplasmic tungstate-binding protein TupA, component of an ABC-type transporter	2998	01547

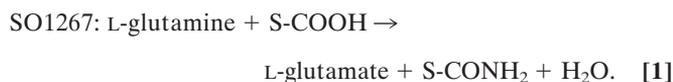
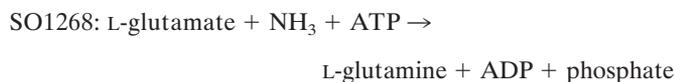
Table 3. Annotations of proteins with well defined biochemical function: Category 2

SO ID no.	Upgraded annotation	COG no.	Pfam no.
SO0265	Cytochrome c-type biogenesis factor CycH	4235	00515
SO0337	Endoribonuclease L-PSP	0251	01042
SO0363	Nucleoside-diphosphate-sugar pyrophosphorylase	1208	—
SO0455	TRAP-type dicarboxylate transporter, permease component with fused DctQM subunit	4666	06808
SO0471	Flavin-dependent dioxygenase	2070	03060
SO0783	Superfamily I DNA and RNA helicase	3972	—
SO1007	Na ⁺ /H ⁺ antiporter NhaC	1757	03553
SO1267	Glutamine synthetase-associated glutamine amidotransferase	2071	00117
SO1742	3-oxoacyl-acyl-carrier-protein	0332	00195
SO1981	Nicotinic acid phosphoribosyltransferase	1488	04095
SO3051	Mo-dependent oxidoreductase maturation factor	1975	—
SO3542	Phosphoketolase	3957	03894
SO3667	Heme iron utilization protein HugZ	0748	01243
SO3668	Heme iron utilization protein HugX	3721	06228
SO4227	S-adenosylmethionine-dependent methyltransferase MraW, involved in cell division	0275	01795
SO4690	Undecaprenyl phosphate-sugar: lipid A glycosyltransferase	1807	02366

ization was determined for only 3% of the hypothetical proteins of interest.

Homology-Based Searches and Biochemical Functions. Homologs were detected by exhaustive PSI-BLAST and RPS-BLAST searches (36, 37) for a significant fraction (97%) of the expressed conserved proteins. Even when these homologs had experimentally characterized functions, the relatively low degree of sequence identity made such functional annotations somewhat uncertain. Nevertheless, in many cases formerly hypothetical proteins could be confidently assigned to a known protein family by using the domain-specific profile search tools provided by the Conserved Domain, COG, or Pfam databases. This approach resulted in a dramatic improvement in the search sensitivity and allowed well defined, or at least general, biochemical functional assignments [Tables 1 (categories 2 and 3) 3, and 5, respectively]. For example, even with the presence of a clearly defined biochemical activity for protein SO0471 (Flavin-dependent dioxygenase), its exact substrate specificity remained uncertain, resulting in a category 2 assignment for this gene (Table 3).

In one notable example, *S. oneidensis* gene SO1267, predicted to be a glutamine amidotransferase, was found in the same operon with a gene encoding glutamine synthetase (SO1268). Clearly, the combined activities of these two enzymes provides for the conversion of ammonia into an amide group of some unknown substrate at the expense of one ATP molecule, whereas glutamate is recycled:



Unfortunately, the exact nature of the substrate *S* remains unknown, resulting in assignment of SO1267 (Glutamine synthetase-associated glutamine amidotransferase) to category 2 (Table 3).

Those proteins that could not be assigned to known protein families could sometimes be functionally annotated based on conserved protein sequence motifs or by using other data from the literature. Thus, for the pair of proteins SO0110 and SO0578, both annotated as “metalloprotease,” our revised annotations distinguished the two. SO0110 was reannotated as “metalloprotease, family M48” (Table 5, category 3), which corresponds to a known protein family (COG0501). In contrast, SO0578 was reannotated as “Zn-dependent metalloprotease domain-containing protein,” based on the presence of a signal peptide and a conserved “HEXxH” Zn²⁺-binding motif but only a limited similarity to COG1913 (Table 6, category 4).

General Biological Activity Prediction and Certain Functional Insights.

Combining all sources of information in public databases and experimental data can be used to predict general biological function [Tables 1 (category 4) and 5] or at least to get certain functional insights [Tables 1 (category 5) and 7] for many expressed hypothetical proteins. To this end, two genome context data analysis methods were implemented. The first approach focused on operons and chromosomal clusters of genes conserved between diverse species (SEED). The second method focused on predicting regulatory binding sites (38) and is summarized in Table 10, which is published as supporting information on the PNAS web site. Information regarding protein-protein interactions was also used but with limited success, as was the case in the recent pilot study (11).

In contrast, structural genomics data made an important complementary contribution toward annotating hypothetical genes. As far as we are aware, *S. oneidensis* has not been a subject of a focused structural genomics project, so very few solved structures have been

deposited in the Protein Data Bank (33). Nonetheless, information on structural domains from the Structural Classification of Proteins-based SUPERFAMILY database (39) searches by using DARWIN were valuable for the functional assignments for ≈200 genes (6). In many cases, this structure-based information provided additional supporting evidence for annotations obtained through other means, typically from similarities to proteins with known structure (Table 6, category 4). One such example is SO1195, earlier annotated as a “putative RNA-binding protein (30).” We were able to identify a closely related homolog with known 3D structure and further predict its regulatory binding site (Table 10). As a result, SO1195 has been reannotated as “RNA-binding protein with a KH domain” (Table 6, category 4). To the extent possible at this point, these structure-based results seem to accurately reflect the somewhat-limited contribution of structural genomics to the understanding of protein function on the genome scale (10, 11, 42).

Finally, combining all available data provided only certain functional insights for numerous proteins of interest. For example, no clear functional annotation was obtained for SO0066 and SO0308, so they have been annotated as “conserved extracellular α -helical protein” and “DUF1212 family domain-containing membrane protein with eight transmembrane segments,” respectively [Tables 1 (category 5) and 7]. Altogether, 256 proteins with significantly or modestly improved annotations were identified (categories 1–5; 48%) and are listed in Tables 2, 3, and 5–7.

Proteins Expressed Under Certain Growth Condition(s). Despite comprehensive analysis and careful curation performed by several groups of researchers, we were unable to significantly improve annotations for 52% of the expressed hypothetical proteins. These proteins are listed in Tables 8 and 9 (categories 6 and 7). The key characteristic of the sixth and seventh categories is the distinction between proteins that are conserved in different phylogenetic lineages or at least different genera [Tables 1 (category 6) and 8], and organism- or genus-specific (herein *Shewanella*-specific) proteins [Tables 1 (category 7) and 9]. For some of these proteins, SO0046 for example, numerous homologs have been identified in diverse organisms, resulting in annotation of this gene as “conserved expressed protein” (Table 8, category 6). For some other proteins, homolog(s) were found only in *Shewanella* species. For example, the only homolog of SOA0042 was found in *Shewanella* SAR-1, whose genome sequence was obtained by assembly of Sargasso Sea microbial community DNA sequences (12). Hence, SOA0042 has been annotated as “plasmid-encoded expressed protein in *Shewanella*” (Table 8, category 7).

***S. oneidensis* Physiological Capabilities.** To further illustrate improvements in our understanding of how the *S. oneidensis* proteins relate to its physiological capabilities and metabolism, we analyzed new functional assignments of former hypothetical genes. Former hypothetical proteins reannotated in this study were classified according to the COG functional categories by comparing each protein to the COG-based profiles with RPS-BLAST (37) with default parameters. Then, these functional category assignments were manually validated by independent experts and summarized in Table 11, which is published as supporting information on the PNAS web site. We compared these assignments against the COG functional category assignments made for all originally annotated proteins. The distributions of the functional categories among former hypothetical proteins and previously characterized proteins were found to be significantly different ($P = 0.0005$; Table 11). For example, fewer DNA replication, recombination, and repair proteins (COG functional category L) were found among the former hypothetical genes. This result was expected because these proteins are among the most conserved ones and are therefore easier to characterize by standard similarity approaches. In contrast, proteins involved in secondary metabolism (COG category Q) and post-translational modification (COG category O) as well as outer

Table 4. Annotations of representative energy-related ion transport and membrane proteins

SO ID no.	Annotation category	COG no.	COG category	Upgraded annotation
SO0311	3	1032	C	FeS center-containing oxidoreductase
SO0363	2	1210	M	Nucleoside-diphosphate-sugar pyrophosphorylase
SO1007	3	1757	C	Na ⁺ /H ⁺ antiporter NhaC
SO1309	3	1629	P	TonB-dependent outer membrane receptor
SO1520	3	0247	C	FeS center-containing oxidoreductase
SO1622	3	0716	C	Flavodoxin; chromosomally linked to PTS system glucose transporter PtsG
SO2512	3	4659	C	Na ⁺ -translocating ubiquinone oxidoreductase, subunit RnfG
SO2523	3	1629	P	TonB-dependent outer membrane receptor; chromosomally linked to phytase
SO3340	1	0668	M	Mechanosensitive ion channel protein MscS
SO3514	3	1629	P	TonB-dependent outer membrane receptor
SO3667	2	0748	P	Heme iron utilization protein HugZ
SO3668	2	3721	P	Heme iron utilization protein HugX
SO4227	2	0275	M	S-adenosylmethionine-dependent methyltransferase MraW, involved in cell division
SO4680	1	1887	M	CDP-glycerol:poly(glycerophosphate) glycerophosphotransferase
SO4690	2	1807	M	Undecaprenyl phosphate-sugar: lipid A glycosyltransferase

COG categories: C, energy production and conversion; M, cell envelope biogenesis, outer membrane; and P, inorganic ion transport and metabolism.

membrane proteins (COG category M) were more common among the former hypothetical genes (Table 11).

It is worth noting that *S. oneidensis* gene distribution among general functional categories is consistent with that observed for other bacteria with relatively large genomes (43, 44). Indeed, *S. oneidensis* appears to be enriched in genes that code for transcription and its regulation (COG functional category K), signal transduction (COG category T), secondary metabolism (COG category Q), cell motility (COG category N), and energy production and conversion (COG category C) systems, consistent with the data of a recent study (44). For example, several proteins involved in energy production and conversion were previously identified and annotated, including Na⁺/H⁺ antiporters and ubiquinone oxidoreductases. Our analyses revealed additional proteins in this class (SO1007, “Na⁺/H⁺ antiporter NhaC,” and SO2512, “Na⁺-translocating ubiquinone oxidoreductase, subunit RnfG”; see Table 4). In addition to the sole flavodoxin (SO2330) found in the original annotation (3), this study added another one (SO1622, “Flavodoxin; chromosomally linked to PTS system glucose transporter PtsG”). We also confirmed the expression of two formerly hypothetical proteins that were subsequently reannotated as FeS center-containing oxidoreductases (SO0311 and SO1520; Table 4). For SO1520, an upstream regulatory binding site was also detected (Table 10).

The unique respiratory versatility of *S. oneidensis* may be traced not only to its exceptionally high number of *c*-type cytochromes (currently estimated to be 42; see ref. 4) but also to the existence of several copies of numerous specialized genes, as noted above. Additionally, we were able to find new genes that code for ion transport proteins (COG category P). For example, this study detected two copies of heme iron utilization proteins (HugZ, SO3667; and HugX, SO3668; Table 4), neither of which was previously recognized. We also were able to improve the annotation of several outer membrane proteins (COG category M), previously described as hypothetical genes. Among others, they include three TonB-dependent outer membrane receptors (SO1309, SO2523, and SO3514; Table 4).

Discussion

Approximately 50–70% of the proteins encoded in any given genome are homologous to genes already annotated in current databases, but each newly sequenced genome adds hundreds to

thousands of uncharacterized genes (7–12). This study focused on *S. oneidensis* strain MR-1 that has recently begun serving as a model microorganism for determining the genetic basis of the metabolic respiratory versatility in metal-reducing *Shewanella* (3, 4, 14, 16, 17, 23). Transcriptome and proteome analyses resulted in the identification of 538 hypothetical genes as expressed in *S. oneidensis* cells (Fig. 1). Special emphasis was placed on robust, reproducible, and statistically validated expression results rather than optimizing coverage. It is becoming an imperative for any high-throughput, whole-genome, integrative study to implement proper experimental designs for different types of analyses and develop standards and statistical models, tailored specifically toward different platforms, approaches, and data types (40). These methods, in turn, will help quantify the certainty of identifications enabling researchers to differentiate between clear-cut cases (conservative estimates), less-certain findings or indeterminate data, and clearly noisy data, allowing them to more easily extract biologically relevant information (11, 22, 28, 40).

A seven-category schema for classifying the annotation level of proteins was developed and applied (Table 1) by using experimental data from transcriptome and proteome analyses and a variety of publicly available analysis tools to achieve an improved annotation of the targeted genes. We were also able to identify homologs for nearly all (520, 97% of 538) expressed proteins. The existence of such a large and uncharacterized group of genes that are conserved among a variety of organisms is disconcerting in that it highlights major gaps in our understanding of basic biology (2, 7–13, 22). Using a tiered-based annotation approach, this study significantly or modestly improved annotations for 256 proteins (categories 1–5; 48% of 538). An important advantage of this tiered approach is its ability to prioritize the functional interrogation and validation of these proteins. Formerly hypothetical proteins assigned to categories 2–4 (168, 31% of 538) will have high priority for inclusion in screens for characterization and validation of specific function(s). Only a small number of proteins, i.e., those in category 1 with confident assignments of exact biochemical function (16, 3% of 538), will not require additional analyses.

The new functional annotations for these proteins will help in our understanding of *S. oneidensis* biology and enhance our ability to deduce the complex networks and pathways responsible for this organism’s metabolic versatility through global expression analyses. The newly annotated proteins are predicted to be involved in

fundamental metabolic processes, including energy conversion and ion transport, as well as in biosynthesis of specific cell components such as membranes (Table 4). The extensive respiratory versatility of *S. oneidensis* is likely due, at least in part, to its exceptionally high number of *c*-type cytochromes (4). Additionally, the *S. oneidensis* genome contains one of the highest numbers of sensor proteins among all sequenced prokaryotes (3, 45). The ensemble of these sensor proteins includes, among others, 45 histidine kinases, 26 methyl-carrier chemotaxis proteins, and 52 diguanylate cyclase domain proteins (45). The number of two-component response regulators in the *S. oneidensis* genome is also among the highest observed among sequenced prokaryotes. The information obtained in this study is ultimately expected to lead to a deeper understanding of how *S. oneidensis* is able to sense and use such a wide range of electron acceptors for anaerobic respiration, including solid phase metal oxides such as Fe₂O₃, Mn₂O₃, and MnO₂ (1).

Several limitations of the approach used in this study are worth noting. When both high-throughput gene and protein expression data were combined by using stringent criteria, the resulting overlapping dataset (538 proteins) was highly correlated (91%) with the protein expression dataset alone (592 proteins). Careful examination of 53 “dropped” genes showed that (i) some of these genes were not included on the arrays (e.g., SO3039), and (ii) half of these genes were expressed as mRNAs (when less stringent criteria were used), resulting in the overlapping dataset of 566 (96% of 592). Clearly, the protein expression dataset was found to be the most critical in this study. Nongel-based (MS) proteomics methods are highly preferred with regard to protein identifications, as supported by both recent estimates of protein identifications obtained for several microorganisms by applying different proteomics platforms (e.g., 22). The data obtained herein lead to the same observation: 95% of protein identifications were obtained by nongel-based methods. Altogether, with the above limitations applied, when analyzing genes

encoding proteins, MS-based proteomics approaches were found to be most effective in this study.

The increasing amount of data obtained from genome sequencing projects and growing databases of sequence profiles enhance the sensitivity of programs such as PSI-BLAST and RPS-BLAST. These factors, along with a variety of comparative genomic approaches, allow researchers to significantly improve genome annotation. This study demonstrated how these approaches and expression data were integrated to gain additional insight into the functions of >250 previously uncharacterized *S. oneidensis* proteins.

As the list of the hypothetical genes continues to grow, the challenge they pose to our understanding of how genomes actually function also increases (9–11). Integrative studies that combine expression analysis, computational biology, comparative genomics, and careful curation can help to achieve a more comprehensive and accurate annotation of sequenced genomes. These factors, in turn, will create reasonable and verifiable hypotheses for further experimental work toward better understanding and prediction of cellular function and physiology.

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