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Global Transcriptome Analysis of the Cold Shock Response of *Shewanella oneidensis* MR-1 and Mutational Analysis of Its Classical Cold Shock Proteins‡

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This study presents a global transcriptional analysis of the cold shock response of *Shewanella oneidensis* MR-1 after a temperature downshift from 30°C to 8 or 15°C based on time series microarray experiments. More than 700 genes were found to be significantly affected ($P \leq 0.05$) upon cold shock challenge, especially at 8°C. The temporal gene expression patterns of the classical cold shock genes varied, and only some of them, most notably so1648 and so2787, were differentially regulated in response to a temperature downshift. The global response of *S. oneidensis* to cold shock was also characterized by the up-regulation of genes encoding membrane proteins, DNA metabolism and translation apparatus components, metabolic proteins, regulatory proteins, and hypothetical proteins. Most of the metabolic proteins affected are involved in catalytic processes that generate NADH or NADPH. Mutational analyses confirmed that the small cold shock proteins, So1648 and So2787, are involved in the cold shock response of *S. oneidensis*. The analyses also indicated that So1648 may function only at very low temperatures.

Temperature is a common environmental factor, and virtually all organisms elicit a cellular response to an increase or decrease in temperature. Cold shock has a profound impact on cell growth by influencing ribosomal synthesis, cytoplasmic membrane composition and fluidity, protein synthesis, and solute uptake (41). In recent years, most of the studies of the bacterial cold shock response have focused on *Escherichia coli* (27). When the culture temperature of exponentially growing *E. coli* cells is shifted from 37°C to a temperature below 20°C, there is growth arrest before resumption of cell growth (22). A set of proteins called cold-induced proteins are transiently induced at very high levels during the growth arrest period (19, 52). Cold-induced proteins, previously called cold shock proteins (CSPs), are predominantly small, acidic proteins (27).

CSPs are defined on the basis of their highly conserved sequences containing a prototype of the RNA-binding cold shock domain (CSD), which is highly conserved from bacteria to humans (51). CSPs constitute a widespread family of small (approximately 70 amino acids [aa]), nucleic acid-binding proteins. This protein family has been identified in almost all psychrotrophic, mesophilic, thermophilic, and hyperthermophilic bacteria examined so far, despite copies of CSPs varying from one in *Haemophilus influenzae* to nine in *E. coli* (20, 41).

The facultative anaerobe *Shewanella oneidensis* MR-1, a dissimilatory metal-reducing bacterium, has been extensively studied because of its wide distribution, metabolic versatility, and environmental importance (49). Unlike *E. coli*, MR-1 is psychrotolerant because of its ability to grow at 5°C or lower, according to the revised definition of Morita and Moyer (37). In recent years, more attention has been paid to the genetic structure, gene functions, and regulatory networks directly linked to its dissimilatory metal reduction pathways. However, mechanisms by which this bacterium survives and adapts to various environmentally relevant stresses remain largely unexplored, with the exception of a few recent studies (16, 35).

Previously, we have investigated the response of *S. oneidensis* to temperature upshift and found that MR-1 elicits a heat shock response similar to that of *E. coli* (16). Although low-temperature growth of *S. oneidensis* was reported recently, no study has been conducted to investigate the bacterial cold shock response at the whole-transcriptome level (1). Here, we used whole-genome microarrays to investigate temporal gene expression profiles in response to a temperature downshift from 30°C to 8 or 15°C over a period of 160 min. Analysis of the transcriptional profile after cold shock clearly revealed significant differences in the expression of CSPs between *S. oneidensis* and *E. coli*. Certain genes encoding membrane proteins and components of the translation apparatus displayed the most dramatic up-regulation. Interestingly, most of the induced genes encoding metabolic enzymes have previously been shown to be induced by heat shock (16). Mutational analysis of CSPs revealed that So1648 may be the only CSP working at low temperatures. It is anticipated that knowledge of global transcriptional responses to environmental stresses will be useful in elucidating the specific mechanisms enabling cellular survival and adaptation to stress.

Characteristics of *S. oneidensis* growth at low temperatures. All *S. oneidensis* strains were grown in Luria-Bertani (LB) medium (Difco, Detroit, MI) at 30°C to assay their optimal

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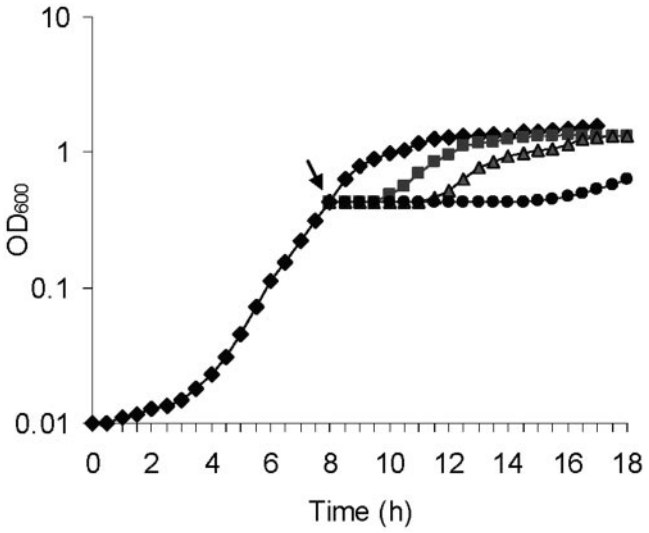

FIG. 1. Growth of *S. oneidensis* MR-1 at 30°C (◆) and after cold shock to 15 (■), 8 (▲), or 5°C (●). The arrow indicates the time point of cold shock (OD_{600} of 0.43).

growth kinetics by using a Bioscreen C Labsystems (Helsinki, Finland) microbiology reader with multiwell disposable microtiter plates according to the manufacturer's instructions. Cold shock experiments were carried out in flasks aerobically. Growth was measured every 30 min for the physiological optimal (nonstress) conditions and every 4 h for the stress conditions (5, 8, or 15°C). For growth measurements, 5°C was chosen for investigating the psychrotolerance of *S. oneidensis*.

The growth of *S. oneidensis* MR-1 was characterized at 30°C and after temperature downshifts to 5, 8, or 15°C. *S. oneidensis* responded to low temperatures in terms of growth similar to *E. coli* and *Vibrio cholerae*, the only organism with extensive regions of gene synteny similar to those of *S. oneidensis* (24). Growth paused immediately and then resumed after a certain lag period when logarithmically growing *S. oneidensis* cells (an optical density at 600 nm [OD_{600}] of 0.43) were exposed to cold temperatures, indicating that these low temperatures are growth permissive for *S. oneidensis* (Fig. 1). The general observation is that the lower the exposure temperature, the longer the lag time observed (11, 18). The lag times for *S. oneidensis* were 8 h, 150 min, and 90 min for the treatment temperatures of 5, 8, and 15°C, respectively. However, in contrast to *E. coli* and *V. cholerae* cells, *S. oneidensis* cells were able to resume growth after being shifted to 5°C, indicating that *S. oneidensis* has higher psychrotolerance. After the lag period, growth resumed with a longer generation time. In addition, no differences in the final cell density were observed after cells reached the stationary phase.

Experimental design, sampling, hybridization, and assessment of array data quality. For temporal transcriptome analysis of cold-shocked *S. oneidensis* cells, samples were taken after a temperature downshift from 30°C to 15 or 8°C. A temperature of 15°C was chosen for this study based on standardized cold shock experimental conditions for mesophilic bacteria (52). Moreover, since the change in temperature between the initial growth temperature and the cold shock temperature is important and not the cold shock temperature per se, we chose 8°C for this study so that the change of temperature was 22°C, which is generally regarded as standard for cold shock study of bacteria (26).

Cells for microarray analyses were prepared as described previously (16). In brief, a 25-ml aliquot of mid-log-phase cells (OD_{600} of 0.59) was transferred to a 250-ml flask precooled to 8 or 15°C and then incubated in an 8 or 15°C shaking water bath. Samples for microarray analysis were taken at 0 (control), 5, 10, 20, 40, 80, and 160 min, pelleted by centrifugation at maximum speed in a 5415R centrifuge (Eppendorf, Germany) for 10 s, and placed in liquid nitrogen.

Total RNA extraction and cDNA labeling have been described elsewhere, with slight modifications (48). RNA from the control samples was fluorescently labeled with Cy3, and that from the stressed samples was labeled with Cy5. A study of the necessity of technical replicates, including dye swap, was conducted in our laboratory when the protocol with genomic DNA as a cohybridization standard was introduced (53). Results revealed that dye bias was consistent across samples and can be corrected by normalization. Since biological replicates account for both technical and biological variations in the microarray assay, it is generally the consensus that more biological replicates provide greater power (4). In this study, five biological replicates were carried out for each time point without dye swap. Construction of *S. oneidensis* whole-genome microarrays, hybridization, scanning, and data analysis have been described previously (16).

The quality of the array data was assessed using two methods. First, we conducted statistical analyses on genes from operons or on genes picked randomly as described previously (16). A distribution of the absolute difference of normalized log ratio versus relative gene frequency similar to that obtained in our heat shock study was observed (data not shown). Consistent with our expectation, the genes within the same operon responded more similarly to cold shock conditions than genes randomly selected from the genome, indicating the high quality of the gene expression data. Second, eight open reading frames (ORFs) showing high induction after cold shock in microarray analysis were subjected to real-time quantitative reverse transcription-PCR with the same RNA samples used in the array hybridizations, according to a protocol described previously (16). The primers used are listed in Table S1 in the supplemental material. A high level of concordance ($R^2 = 0.918$) was observed between microarray and real-time reverse transcription-PCR data despite quantitative differences in the level of change (see Fig. S1 in the supplemental material). This is consistent with many previous reports (13, 16, 35, 50).

General overview of transcriptomic response of *S. oneidensis* to cold shock. Whole-genome DNA microarrays were used to obtain a comprehensive, general description of the molecular response mounted by *S. oneidensis* when challenged by a temperature downshift. In total, 785 (320/465, induced/repressed) and 546 (217/329) genes at 8 and 15°C, respectively, exhibited significant differential expression ($P \leq 0.05$) at a twofold or greater level in at least one of the six time points. These total gene numbers represent about 16.9% (8°C) and 11.7% (15°C) of the 4,648 ORFs spotted on the array. Up to 97% of the genes affected significantly at 15°C were recorded similarly at 8°C. To simplify the analysis, our discussions are focused on

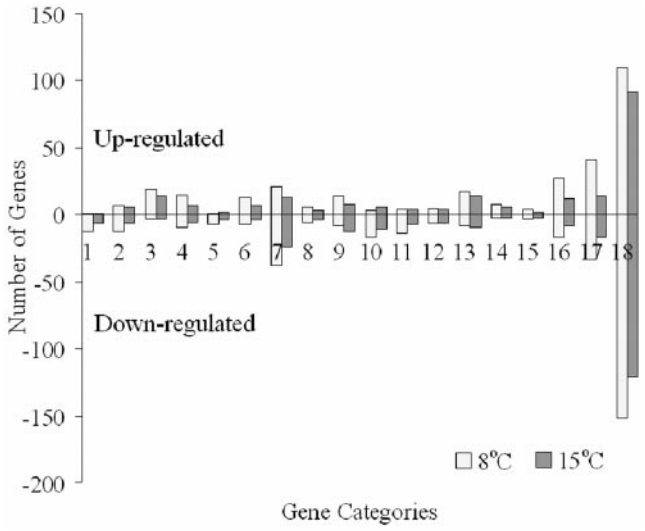

FIG. 2. Differentially expressed genes grouped by functional classification, according to the *S. oneidensis* genome database. Columns: 1, amino acid biosynthesis; 2, biosynthesis of cofactors, prosthetic groups, and carriers; 3, cell envelope; 4, cellular processes; 5, central intermediary metabolism; 6, DNA metabolism; 7, energy metabolism; 8, fatty acid and phospholipid metabolism; 9, other categories; 10, protein fate; 11, protein synthesis; 12, purines, pyrimidines, nucleosides, and nucleotides; 13, regulatory functions; 14, signal transduction; 15, transcription; 16, transport and binding proteins; 17, proteins of unknown function; 18, hypothetical proteins.

the results from the profiling experiments based on the 8°C samples, unless otherwise noted.

Down-regulated genes were dominant in such functional categories as amino acid biosynthesis, protein fate, and protein synthesis, whereas genes encoding cell envelope and transport/binding proteins were induced upon temperature downshifts (Fig. 2). It is interesting that the majority of the genes (~73%) involved in energy metabolism were down-regulated. This is contradictory to our previous results with such stressors as heat shock and salt stress, which showed an increase of gene expression in energy production (16, 35). Genes encoding proteins involved in the biosynthesis of cofactors, prosthetic groups, and carriers, cellular processes, and regulatory functions and functionally unknown proteins displayed similar profiles in terms of the numbers of genes induced and repressed (approximately 20 to 30%) under both conditions. Only a few genes whose products are in central intermediary metabolism, fatty acid and phospholipid metabolism, purines, pyrimidines, nucleosides, and nucleotides were either induced or repressed significantly.

Cold shock proteins of *S. oneidensis*. Extensive studies of the cold shock response of *E. coli* have revealed that more than 30 genes are cold inducible (27). However, these genes in *S. oneidensis* displayed different expression patterns and some were not induced at all upon a decrease in temperature (Fig. 3A). The genes induced included so1203 (*nusA*, N utilization substance protein A; 3.4-fold induction), so1204 (*infB*, translation initiation factor IF-2; 4.4-fold), so1205 (*rbfA*, ribosome-binding factor A; 4.8-fold), so1797 (DNA-binding protein, HU family; 2.6-fold), and so2625 (*infA*, translational initiation factor IF-1; 2.4-fold). The transcription level of these induced genes was up-regulated immediately after temperature treatment, peaked at 10 min under both conditions (8 and 15°C), and then gradually decreased over time. An exception was so3422 (*yfiA-2*, ribosomal subunit interface protein; 4.7-fold induction), whose expression was highly induced throughout the entire treatment period, even at 15°C. The results for these genes are discussed later.

Genomic analysis of *S. oneidensis* revealed three copies of canonical CSPs (So1648, So2787, and CspD) with a size of approximately 70 aa (24), whereas *E. coli* has nine CSPs, with only four (CspA, CspB, CspG, and CspI) that are cold shock inducible, and *V. cholerae* has four (23, 52). The derived amino acid sequence of the gene so1648 displayed high sequence similarities to *E. coli* CspA (identities, 65%; positives, 77%), suggesting that it may be a major CSP in *S. oneidensis*. Microarray results revealed that this gene was highly up-regulated throughout the time course, with a peak induction of 14-fold after 10 min at 8°C. Gene so2787 was barely affected by the temperature downshifts. Consistent with the observation in *E. coli*, gene so2628 (*cspD*) was not induced (54).

Sequence analysis of the *S. oneidensis* genome revealed that two genes encode proteins (So0733, 203 aa; So1732, 224 aa) containing a CSD at their N-terminal end (1 to 68 aa), which is entirely homologous to CSPs, and a region without significant sequence similarity to known proteins. Neither so0733 nor so1732 was found to be induced in this study, and more exploration is needed to determine their roles in the cold shock response of *S. oneidensis*. Intriguingly, such a structure has been found only in eukaryotes, with the exception of *Mycobacterium* spp. (19, 52). While the function of these proteins in *Mycobacterium* remains unknown, a couple of studies of the eukaryotic counterparts have revealed a variety of functions (43, 46). However, none of these functions are related to cold shock response. All these results suggested that the cold shock response in *S. oneidensis* appears to be different in some significant aspects from that in *E. coli*, at least in the case of CSPs.

Membrane-associated proteins and transporters. It is known that bacterial cells increase a proportion of unsaturated membrane lipids to maintain the cell membrane in a biologically functional fluid phase in response to a lowered environmental temperature. Lipopolysaccharide (LPS) is a unique constituent of the bacterial outer membrane that forms a protective barrier around the cell, and its synthesis was reportedly induced at low temperatures (9, 39). Induction of so2088 (lipid A biosynthesis acyltransferase; 2.8-fold induction) and so3179 (lipopolysaccharide biosynthesis polymerase; 2.4-fold), whose predicted products are involved in LPS biosynthesis, has been observed in the course of the treatment (Fig. 3B). The LPS So2088 shares 29% identity and 48% similarity to LpxP from *E. coli*, which is inducible upon a temperature downshift. It is believed that LpxP functions to enhance the outer membrane fluidity after cold shock with the acylation of lipid A with low-melting-point palmitoleate instead of laurate (9). In addition, genes encoding lipoproteins and lipoprotein-releasing proteins, including so1872 (lipoprotein; 2.7-fold induction), so2257 (*lolE*, lipoprotein releasing system transmembrane protein; 2.4-fold), so2258 (*lolD*, lipoprotein releasing system ATP-binding protein; 2.3-fold), so2307 (*lolA*, outer membrane lipoprotein carrier protein; 3.2-fold), so3150 (lipoprotein; 3.5-fold), so3811

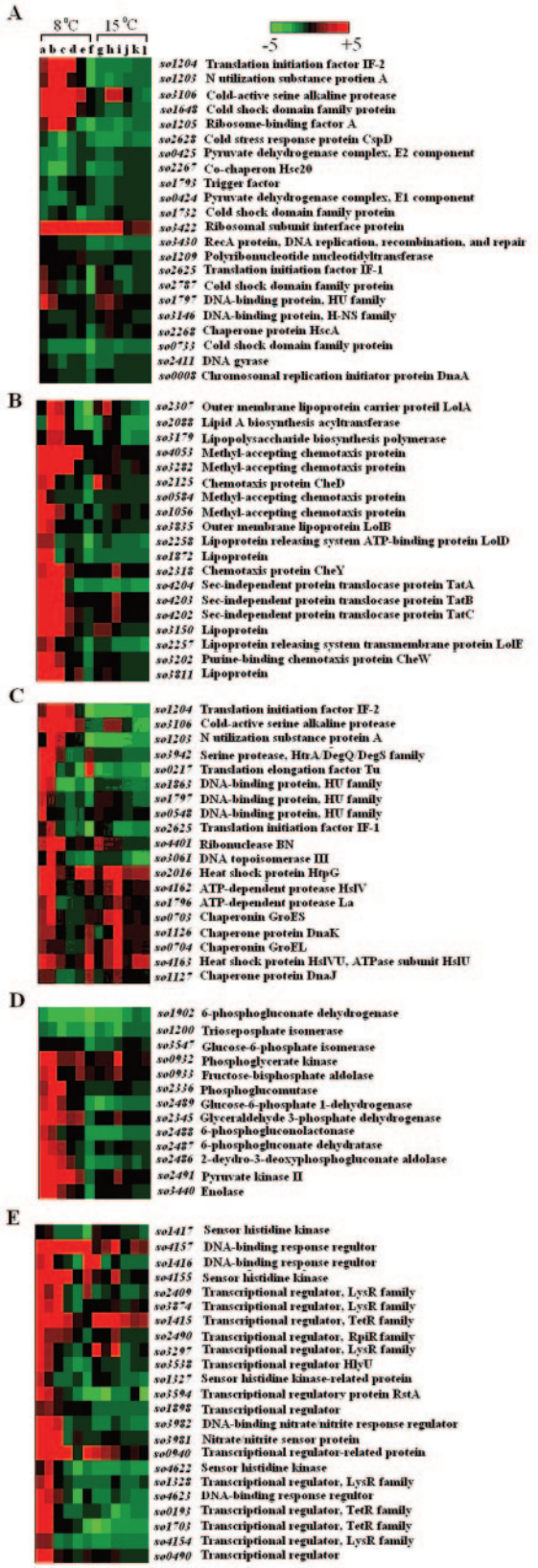

FIG. 3. Expression of the *S. oneidensis* MR-1 genes exhibiting altered mRNA levels upon cold shock. (A) *S. oneidensis* counterparts of cold shock proteins in *E. coli*. (B) Membrane-associated proteins and transporters. (C) DNA-modulating proteins and translation apparatus. (D) Proteins in metabolism. (E) Proteins in regulation. Columns represent time points (a to f, 5 to 160 min at 8°C; g to l, 5 to 160 min at 15°C, as described in the text). Red and green indicate genes that are induced and repressed, respectively.

(lipoprotein; 3.6-fold), and so3835 (*lolB*, outer membrane lipoprotein; 2.7-fold), were induced upon cold shock.

In *E. coli*, the chemotaxis apparatus is involved in molecular thermosensing, and thermal stimuli are sensed by the four closely related transmembrane receptors of monocyte chemoattractant proteins (Tsr, Tar, Trg, and Tap) (38). There are 55 genes encoding the chemotaxis proteins in *S. oneidensis*, and most of them remain uncharacterized. Our analysis revealed that a number of genes encoding components of the chemotaxis apparatus were induced (Fig. 3B). These included so0584 (methyl-accepting chemotaxis protein; 4.3-fold induction), so1056 (methyl-accepting chemotaxis protein; 2.7-fold), so2125 (*cheD-1*, chemotaxis protein; 3.3-fold), so2318 (*cheY-2*, chemotaxis protein; 4.1-fold), so3202 (*cheW-3*, chemotaxis protein; 3.2-fold), so3282 (methyl-accepting chemotaxis protein; 4.2-fold), and so4053 (methyl-accepting chemotaxis protein; 10.6-fold). Due to the high level of sequence similarities among all monocyte chemoattractant proteins, it is impossible to designate the *S. oneidensis* counterparts of *E. coli* Tsr, Tar, Trg, and Tap based on our microarray data only. Further investigation is needed to determine these genes in *S. oneidensis*.

Upon cold shock, it is believed that efficiencies in preprotein processing and translocation are reduced (7). In this study, we observed that the expression of genes encoding components of the general secretory pathway (Sec) and HlyD family secretion proteins was either repressed or unaffected. In contrast, all three genes encoding components of the Sec-independent protein translocase, so4202 (*tatA*; 3.4-fold induction), so4203 (*tatB*; 3.4-fold), and so4204 (*tatC*; 3.6-fold), were induced. Recent studies of the *E. coli* Tat system have shown that the Tat system not only translocates fully folded proteins but also plays a role in the posttranslational biogenesis of integral membrane protein (25). These results suggest that the Sec-independent protein translocases may be important at low temperatures.

DNA-modulating proteins and translation apparatus. One of the deleterious effects of cold shock is stabilization of the secondary structures of RNA and DNA, which may lead to a reduced efficiency of DNA replication, transcription, and translation (41). Therefore, the proteins involved in the initiation step of translation and reactivation of the translation machinery were cold induced (15, 29). However, only a few genes encoding DNA-modulating proteins and the translation apparatus were cold induced in MR-1 (Fig. 3C). Gene so3061 (*topB*; 2.7-fold induction), encoding DNA topoisomerase III, was induced as opposed to *gyrA* in *E. coli* (28, 30). Although these genes encode different types of DNA topoisomerases (TopB, DNA topoisomerase III; GyrA, DNA gyrase), both function to relax a positive superhelical DNA, resulting in the previously observed negative supercoil of DNA after cold shock (10, 32).

S. oneidensis has more than a dozen genes encoding ribonucleases, but only so4401 (*rbn*; 3.2-fold induction) was induced. Interestingly, gene so1209 (*pnp*), whose product is a

well-described cold-inducible RNase in *E. coli*, was not affected by the temperature downshift (55). Another intriguing finding was that none of the genes for nucleoid-associated DNA-binding proteins (H-NS) were induced, although H-NS was well established as a cold-inducible protein in several organisms (14). Furthermore, there were significant differences in both the structure and expression of operons encoding DNA-binding proteins in the HU family between *S. oneidensis* and *E. coli*. The *E. coli* DNA-binding proteins in the HU family, encoded by *hupA* and *hupB*, play an important role during cold adaptation, although only *hupB* was cold inducible (17). Three genes, encoding DNA-binding proteins in the HU family (so0548, 2.8-fold induction; so1797, 2.6-fold; so1863, 2.9-fold) and sharing over 60% sequence identity to both *hupA* and *hupB*, were induced during cold shock in *S. oneidensis*. Together, these results suggest that *S. oneidensis* could employ a different set of proteins to destabilize the secondary RNA and DNA structures.

The elevated mRNA levels of CspA, transcription terminator/antiterminator NusA (so1203 [*nusA*], 3.4-fold induction), translation initiation factors IF-1 (so2625 [*infA*], 2.4-fold) and IF-2 (so1204 [*infB*], 4.4-fold), elongation factor EF-Tu (so0217 [*tufB*], 4.1-fold), and ribosomal subunit interface protein (so3422 [*yfiA-2*], 4.7-fold) in response to cold shock might facilitate gene expression at low temperatures (27). Apart from its general role of chaperoning nascent RNA, CspA from *E. coli* has been shown to act as both a transcriptional activator and an antiterminator, which may explain why a very large number of molecules of CspA are present in the cell (5). EF-Tu mediates the recognition and transport of aminoacyl-tRNAs and their positioning to the A site of the ribosome (8, 21). In *S. oneidensis*, both so0217 (*tufB*) and so0229 (*tufA*) encode elongation factors but only *tufB* was induced upon cold shock. Studies of cold-inducible *E. coli* YfiA demonstrated that the protein interferes with translation elongation by specifically binding to the subunit interface and thus reduces translation errors (2, 3).

Although incorrect protein folding at low temperatures might be less of a problem than it is under heat shock conditions, chaperones and proteases are required to deal with intracellular protein perturbations. Several genes encoding chaperones, including so0703-so0704 (*groES*, 3.2-fold induction; *groEL*, 2.2-fold), so1126-so1127 (*dnaK*, 2.3-fold; *dnaJ*, 2.0-fold), so2016 (*htpG*, 4.0-fold), and so4163 (*hslU*, 2.8-fold), were induced upon cold shock. Although all of these chaperones are canonical heat shock proteins, they have been found to be generally induced in response to a variety of stresses (56). Under unfavorable conditions, misfolded proteins increase significantly. Proteases are needed to remove these detrimental proteins before they accumulate. Four genes encoding proteases, including so1796 (*lon*, 2.7-fold induction), so3106 (*aprE*, 9.5-fold), so3942 (4.5-fold), and so4162 (*hslV*, 4.3-fold), were induced upon cold shock. While *lon*, *hslV*, and so3942 were highly induced upon heat shock, *aprE*, encoding a cold-active serine alkaline protease, was up-regulated only upon cold shock (16). At low temperatures, the cold-active protease AprE is more active than those (such as Lon, HslV, and possibly So3942) derived from mesophilic microorganisms (33). Thus, it is likely that *S. oneidensis* may use AprE, in conjunction with other proteases, to degrade misfolded proteins at very low temperatures. These results, in general, suggested that *S. oneidensis* employs similar sets of proteins to cope with protein perturbations caused by either temperature upshift or downshift.

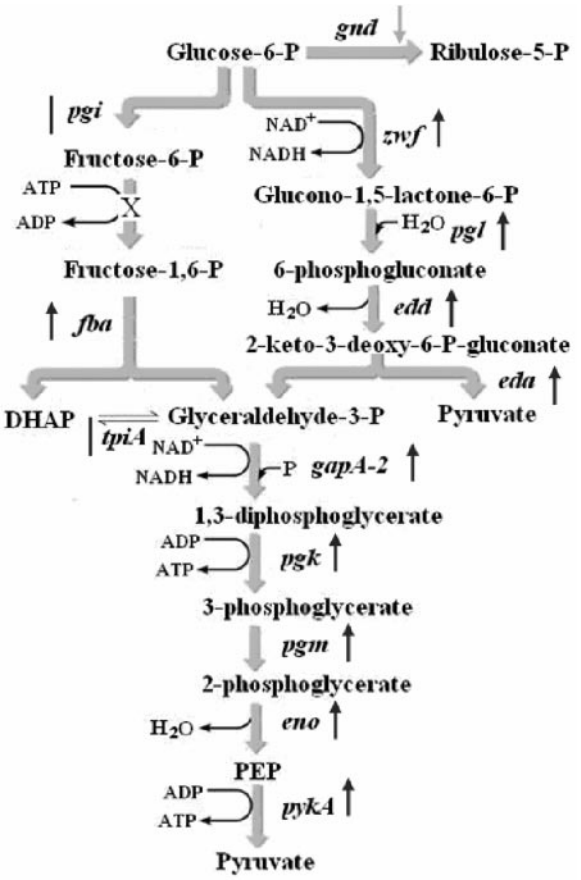

FIG. 4. Glycolysis, Entner-Doudoroff, and associated energy metabolic pathways. Next to the gene symbols, the vertical bars denote no changes in expression, whereas upward arrows and downward arrows denote significant up- and down-regulation in expression, respectively. DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate.

Proteins in metabolism. So far, little information is available concerning the impact of cold stress on cellular metabolism, especially at the genomic level. Two recently published DNA microarray studies of two different cold-shocked *Bacillus subtilis* strains revealed a global down-regulation of metabolically relevant proteins (6, 31). This concept was in agreement with earlier proteomic studies (52). Unfortunately, few DNA microarray studies on the cold shock response in gram-negative bacteria have been reported (42).

The so2486-so2489 operon, which encodes components of the Entner-Doudoroff pathway, was induced upon cold shock as well as heat shock (16). Genes in this operon are so2486 (eda, 2-deydro-3-deoxyphosphogluconate aldolase/4-hydroxy-2-oxoglutarate aldolase; 2.7-fold induction), so2487 (*edd*, 6-phosphogluconate dehydratase; 2.6-fold), so2488 (*pgl*, 6-phosphogluconolactonase; 2.9-fold), and so2489 (*zwf*, glucose-6-phosphate 1-dehydrogenase; 2.4-fold). The pathway not only converts glucose to pyruvate and glyceraldehyde-3-phosphate (G-3-P) but also provides 6-phospho-gluconate for the pentose phosphate pathway (Fig. 3D and 4). More importantly, the pathway enables

S. oneidensis cells to bypass the missing phosphofructokinase step in glycolysis, allowing them to metabolize several sugars (24). The final metabolites of reactions catalyzed by this pathway are pyruvate and G-3-P. G-3-P is likely converted to pyruvate because all of the genes in this pathway were induced, including so0932 (*pgk*, phosphoglycerate kinase; 2.5-fold induction), so2336 (*pgm*, phosphoglucomutase [α-D-glucose phosphate specific]; 2.6-fold), so2345 (*gapA-2*, glyceraldehyde-3-phosphate dehydrogenase; 2.3-fold), so2491 (*pykA*, pyruvate kinase II; 5.0-fold), and so3440 (*eno*, enolase; 2.2-fold).

Pyruvate, one of the most important immediate metabolites, can then be turned into other fermentative end products. In this study, we observed that genes in pathways generating formate and acetyl-coenzyme A (so2912 [*pflB*], formate acetyltransferase; 3.8-fold induction), lactate (so0968 [*ldhA*], D-lactate dehydrogenase; 4.5-fold), and aceto-lactate (so3262 [*ilvB*], acetolactate synthase isozyme I; 2.7-fold) were induced. It has been reported that fermentative end products rather than sugars have been suggested to be the major energy source for *S. oneidensis* (44). It is possible that the fermentative end products, such as pyruvate, formate, lactate, alcohol, glycerol, and acetate, may be used as the major energy source for *S. oneidensis* upon cold shock.

Proteins in regulation. Among the aspects of cold shock response, the regulatory network involved in cold shock responses is the least understood (52). In this study, we identified many regulators that were induced under cold shock conditions. Genes encoding these regulators include so0193, so0490, so0843, so0940, so1327-so1328, so1415, so1416-so1417, so1703, so1898, so2409, so2490, so3297, so3538 (*hlyU*), so3594, so3874, so3981 (*narQ*)-so3982, so4154, so4155-so4157, and so4622-so4623 (Fig. 3E). Unfortunately, most of these regulators are poorly characterized. It is worth noting that four pairs of these induced genes (so1327-so1328, so1416-so1417, so3981 [*narQ*]-so3982, and so4622-so4623) encode two-component systems. These systems, except for that encoded by so3981-so3982, appear to be involved in general stress response since their up-regulation was also observed in our other stress studies (16, 35).

Hypothetical proteins. Based on TIGR annotation (http://www.tigr.org), more than 41% of the *S. oneidensis* ORFs encode hypothetical proteins. In our study, genes for hypothetical proteins comprised 44% of the significantly up-regulated genes upon cold shock. The majority of these genes were from single-gene operons and encode small polypeptides (<80 aa). Little is known about small polypeptides, although some of them were also induced upon other stresses, such as heat shock and salt stress (16, 35). The rest of the induced hypothetical genes were from multigene operons. Operons of which all members displayed consistent expression are listed below for supporting the predicted operon structures. The predicted operon structures were obtained from http://www.microbesonline.org. These operons included so0108-so0109 (5.1- and 3.6-fold induction), so0403-so0404 (3.4- and 3.3-fold), so0727-so0728 (2.8- and 3.2-fold), so1151-so1152 (2.8- and 4.8-fold induction), so1700-so1701 (3.5- and 2.7-fold), so1840-so1841 (2.4- and 2.5-fold), so3719-so3720 (3.3- and 3.0-fold), so4504-so4505 (4.1- and 4.0-fold), and so4521-so4522 (4.6- and 3.3-fold). Surprisingly, none of these operons were induced upon heat shock (16).

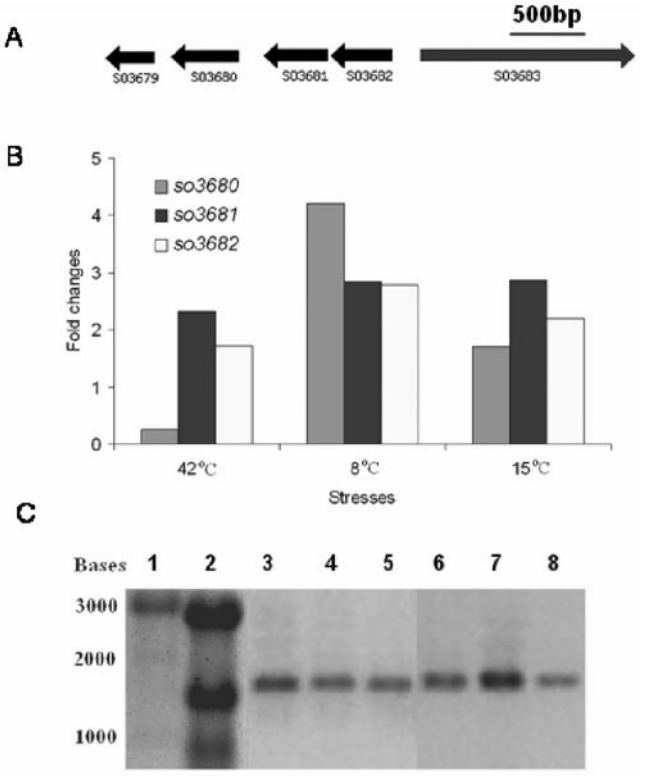

FIG. 5. Microarray and Northern blot analyses of operon so3682-so3680. (A) Organization of so3682 operon and adjacent genes. (B) Expression of so3680, so3681, and so3682 upon heat shock and cold shock. (C) Northern blot analysis results. Lanes 3 to 5 were loaded with RNAs from cold shock samples (5 min of treatment at 8°C) and hybridized with different probes. Lanes 6 to 8 were loaded with RNA from different samples and hybridized with the same probes for so3681. Lanes: 1, RNA ladder; 2, total RNA control (same amount of RNA stained with ethidium bromide); 3, so3682; 4, so3681; 5, so3680; 6, RNA from heat shock samples (5 min of treatment); 7, RNA from cold shock samples (5 min of treatment at 8°C); 8, RNA from control samples. The size of the transcript was about 1.6 kb, as expected. Experiments were repeated three times.

So3682 may be a universal stress protein. The gene encoding the hypothetical protein So3682 is intriguing because it is coexpressed at the transcriptional level along with two genes encoding universal stress proteins (So3681 and So3680) under cold shock conditions (Fig. 5A and B). However, under both heat shock and cold shock conditions, so3682 and so3681 showed a high level of similarity in expression but were significantly different from so3680. To investigate this further, we determined the operon structure by Northern blot analysis. All chemicals used for Northern blot hybridization were obtained from Roche Diagnostics (Roche Diagnostics, Indianapolis, IN), and every step was performed according to the manufacturer's instructions unless otherwise indicated. The primers SO3682F/SO3682R, SO3681F/SO3681R, and SO3680F/SO3680R used to generate probes of ca. 300 bp with specificity for so3682, so3681, and so3680, respectively, are listed in Table S1 in the supplemental material.

As expected, an approximately 1.6-kb transcript was detected with probes for each ORF (Fig. 5C). All these results suggest that the operon contains all three genes, which is in

agreement with the prediction for the *S. oneidensis* MR-1 operon (http://www.microbesonline.org). Furthermore, only one promoter was identified in a 2,000-bp fragment from 500 bp upstream of the first gene (so3682) to the end of the last gene (so3680) by Neural Network Promoter Prediction (http://www.fruitfly.org). The operon structure and coregulated expression patterns also suggest that So3682 may function as a universal stress protein. Although both so3681 and so3680 have been assigned to a universal stress protein functional class based on comparison with UspA from *E. coli* and *Haemophilus influenzae* (http://www.tigr.org), no similar operon structures were obtained by sequence analysis among published microbial genomes with so3681 or so3680 as the target gene (40, 45, 47).

The universal stress protein UspA is a small cytoplasmic bacterial protein whose expression is enhanced severalfold when cellular viability is challenged with any unfavorable agents, such as heat shock, nutrient starvation, stress agents which arrest cell growth, and DNA-damaging agents (34). To confirm the expression of the so3682 operon, Northern blot hybridization analyses using probes with specificity for so3681 were performed. As shown in Fig. 5C, compared to time zero, significantly larger amounts of so3681 RNA were observed in the treated samples, especially heat shock samples, whereas smaller amounts of the mRNA were detected from the control.

Large CSPs are not involved in cold shock response in *S. oneidensis*. As mentioned earlier, five CSP proteins containing a CSD were identified in *S. oneidensis*. Except for So2628 (CspD), whose *E. coli* counterpart has been shown to not be involved in the cold shock response (54), little is known about other CSPs. Although only the so1648 gene was found to be cold inducible in this study, the involvement of other CSPs in cold shock could not be excluded based on the fact that post-transcriptional events play a larger role in the cold shock response of *E. coli* (22).

To determine the role of CSPs in the cold shock response of *S. oneidensis*, we constructed four in-frame deletion mutant strains defective in each of these CSPs. The bacterial strains and plasmids used for this purpose are listed in Table 1. *S. oneidensis* strains were routinely grown in LB medium at 30°C. *E. coli* strains were grown in LB supplemented with 100 μg/ml diaminopimelic acid. Antibiotics were added at the following final concentrations when needed: 25 μg/ml kanamycin and 20 μg/ml tetracycline.

S. oneidensis MR-1 in-frame deletion mutants lacking so0733, so1648, so1732, or so2787 were generated using the *cre-lox* system as described elsewhere (12, 36). The primers used for generating PCR products for mutagenesis and mapping are listed in Table S1 in the supplemental material. Approximately 0.8-kb regions upstream and downstream of these genes were amplified by PCR and cloned into pJK100. The resulting plasmid pJK100 carrying a correct construct was introduced into MR-1 by conjugation from *E. coli* WM3064. Deletion strains of *S. oneidensis* MR-1 were confirmed by PCR with several sets of primers, including a pair targeting the kanamycin cassette (see Table S1 in the supplemental material). Removal of the kanamycin cassette and the helper plasmid was performed as described elsewhere (36). Final verification of all deletion mutations was performed using DNA sequencing.

Compared to those of the wild-type strain, the growth and final cell densities of these strains were not affected by the mutation at 30°C (Fig. 1 and Fig. 6A). When cells of these strains were grown at 15°C, however, two mutant strains (HG1732 and HG2787) displayed statistically significant differences ($P < 0.05$) in maximum cell density (Fig. 6A). Under cold shock condition 3 (15°C), the maximum cell density profile was similar to that observed under condition 2 (Fig. 6A). Strain HG2787 was found to have a significantly lower growth rate than all other mutant strains which grew similarly to the wild-type strain (Fig. 6A and B). In addition to the higher maximum cell density observed, strain HG1732 displayed a shorter lag time. At a much lower shock temperature, both the growth and maximum cell density of strain HG1648 were greatly impaired while strain HG2787 responded similarly to 15°C in terms of maximum cell density (Fig. 6A and C). Interestingly, strain HG2787 displayed a growth rate similar to those of the wild-type, HG733, and HG1732 strains. However, strain HG1732 resumed growth faster and reached the highest maximum cell density.

The growth of the wild-type and all mutant strains under anaerobic conditions was also examined under both normal and cold shock temperatures. Anaerobic growth was assayed in 24-ml anaerobic culture tubes (Bellco Glass, Inc.) filled with 10 ml of LB and sealed with butyl rubber stoppers in an N_2 atmosphere. Filter-sterilized terminal electron donor (lactate) and acceptor (fumarate) were added at the following final concentrations: 20 mM lactate and 20 mM fumarate. No significant differences in maximum cell densities and growth rates were observed for these strains at 30°C (data not shown). On the contrary, both growth rates and maximum cell densities differed among tested strains under cold shock conditions (Fig. 6A and D). Strains HG733 and HG1732 appeared not to be

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. coli strain		
WM3064	Cloning and donor strain for conjugation; Δ*dapA*	Lab stock
S. oneidensis strains		
MR-1	Wild type	Lab stock
HG733	MR-1:Δso0733	This study
HG1648	MR-1:Δso1648	This study
HG1732	MR-1:Δso1732	This study
HG2787	MR-1:Δso2787	This study
Plasmids		
pJK100	Allelic-exchange vector	12
pJK100-0733	pJK100 containing deletion structure for so0733; used to make HG733	This study
pJK100-1648	pJK100 containing deletion structure for so1648; used to make HG1648	This study
pJK100-1732	pJK100 containing deletion structure for so1732; used to make HG1732	This study
pJK100-2878	pJK100 containing deletion structure for so2787; used to make HG2787	This study
pCM157	*cre* expression vector	12

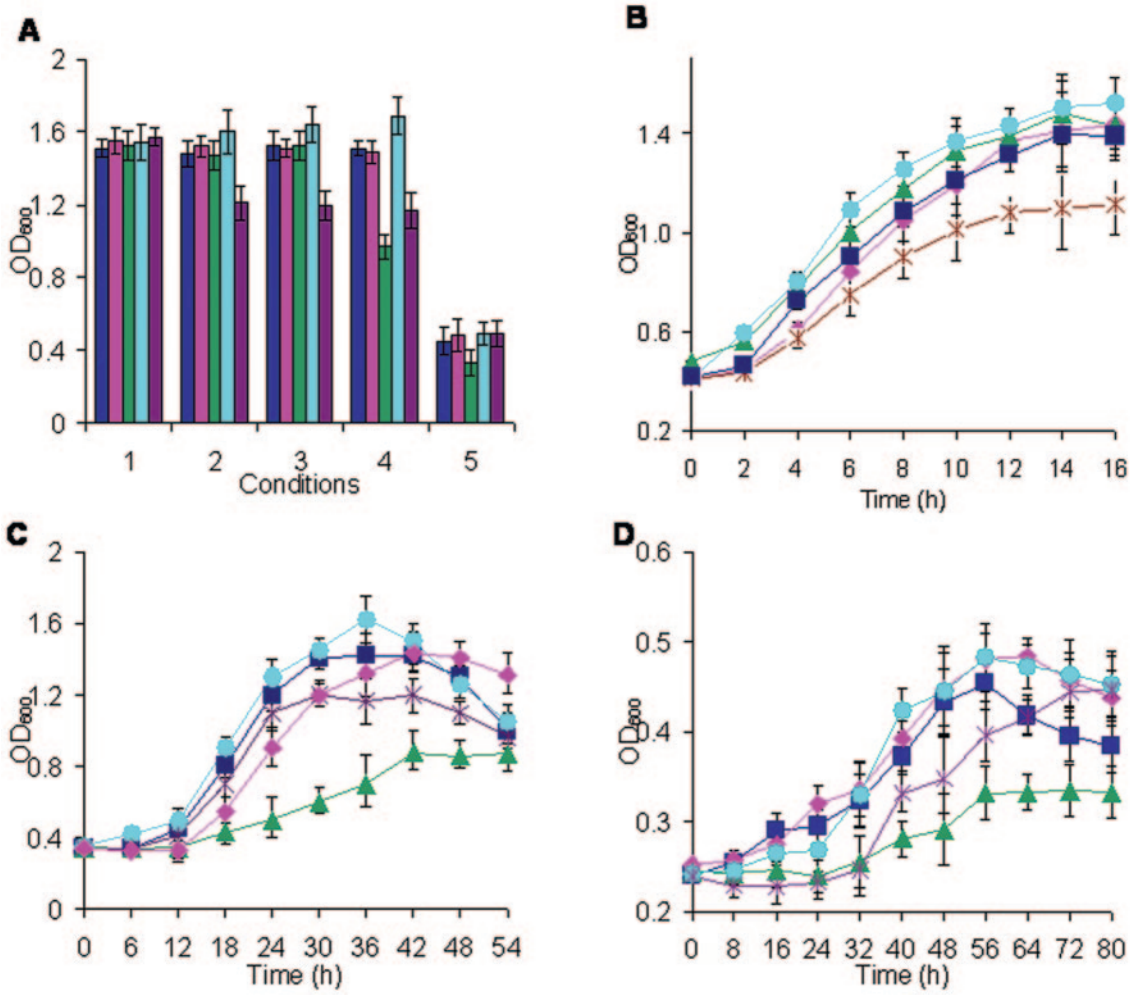

FIG. 6. Characteristics of *S. oneidensis* MR-1 and mutant strains under various conditions. For all panels, strains are shown by color and/or symbol: MR-1, dark blue (■); HG733, pink (◆); HG1648, green (▲); HG1732, light blue (●); and HG2787, purple (x). (A) Maximum cell density of the tested strains under various conditions: 1, grown at 30°C; 2, grown at 15°C; 3, grown at 30°C to the exponential phase and then transferred to 15°C; 4, grown at 30°C to the exponential phase and then transferred to 5°C; and 5, grown at 30°C to the exponential phase and then transferred to 5°C under anaerobic conditions. (B) Growth of tested strains under condition 3. (C) Growth of tested stains under condition 4. (D) Growth of tested strains under condition 5.

affected by the mutations in terms of these two physiological features. Strain HG2787 had an extended lag time compared to growth under aerobic conditions, while it displayed an unimpaired growth rate and maximum cell density. Similar to that observed under aerobic conditions, strain HG1648 had the lowest growth rate and maximum cell density.

Characterization of these deletion mutants revealed three major findings. First, large CSPs (So0733 and So1732) may not be involved in the cold shock response of *S. oneidensis*. In fact, So1732 appeared to have a negative effect on the cellular response at low temperatures. Second, gene so1648, which was highly induced in transcriptional analysis, may encode a protein that is functional only at very low temperatures. We believe that it plays a role similar to that of *E. coli* CspA, but further investigation is still needed. The other small CSP (So2787) may be involved in *S. oneidensis* cold shock response only when the shock is milder. Third, anaerobic cold shock analysis indicated that oxygen tension does not appear to influence the cellular response to cold shock.

Conclusion. Whole-genome DNA microarrays were utilized in this study to probe global gene expression in *S. oneidensis* associated with temperature downshifts. Expression profiles indicated that cold shock has a pleiotropic effect on the *S. oneidensis* transcriptome. The cold shock and heat shock responses of *S. oneidensis* shared a couple of common features, including the employment of a similar set of proteins to manage energy production and protein damage. Among genes encoding CSD-containing CSPs, only so1648 was found to be transcriptionally induced. Mutational analysis revealed that So1732 may retard the bacterial cold shock response, while So1648 is very likely to be the only one working at low temperatures. In terms of CSPs, the cold shock response of *S. oneidensis* is different from that in *E. coli*. This study also revealed that *S. oneidensis* may employ a different set of pro-

teins to cope with the stabilization of secondary RNA and DNA structures compared to that of *E. coli*.

Microarray data accession number. The data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE4489.

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