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Transcriptome Analysis of *Shewanella oneidensis* MR-1 in Response to Elevated Salt Conditions

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Whole-genomic expression patterns were examined in *Shewanella oneidensis* cells exposed to elevated sodium chloride. Genes involved in Na⁺ extrusion and glutamate biosynthesis were significantly up-regulated, and the majority of chemotaxis/motility-related genes were significantly down-regulated. The data also suggested an important role for metabolic adjustment in salt stress adaptation in *S. oneidensis*.

Shewanella species inhabit diverse environments, including spoiled food (11) and infected animals (35), deep-sea and freshwater lake sediments (8, 45, 54), and oilfield waste sites (44). *Shewanella oneidensis* MR-1, a facultative, gram-negative bacterium, was isolated from sediments of Lake Oneida in New York (32). The bacterium can anaerobically respire numerous organic compounds, including fumarate and dimethyl sulfoxide (28), as well as reduce metals such as Fe(III), Mn(IV), Cr(VI), and U(VI) (22, 29, 32). Because of the respiratory versatility, which may be exploited for immobilization of environmental pollutants (i.e., chromium and uranium) in soil and groundwater, the metal-reducing capabilities of *Shewanella* spp. have been intensively investigated (6, 14, 15, 26, 30, 33, 39).

The MR-1 genome was recently sequenced (16), and some fundamental similarities and disparities between MR-1 and other sequenced bacteria have been observed (16). To experimentally probe the genomic response of *S. oneidensis* to various physiologically relevant environmental stresses, a whole-genome cDNA microarray for MR-1 was constructed in this laboratory. In this study, we used this cDNA microarray to profile transcriptional responses of MR-1 to elevated sodium salt stress. The results indicated that the expression of the genes involved in osmolyte protection, cation efflux/influx, motility, and electron transport were significantly altered.

MR-1 requires a relatively high salt concentration for optimal growth. Many *Shewanella* species have been isolated from marine environments, whereas some, like MR-1, have been isolated from freshwater environments (36, 39). To understand how various salt concentrations impact the growth of *S. oneidensis*, MR-1 cells were cultivated in triplicates in MR2A medium (12) containing different amounts of NaCl (ranging in

concentration from 0 to 0.6 M) at 30°C under aerobic conditions (shake flasks, 120 rpm). Growth curves (Fig. 1) indicated that (i) the growth rate increased slightly with additional NaCl levels up to 0.4 M, (ii) cells grown in the presence of 0.4 M NaCl entered stationary-phase growth at a lower optical density (OD) than cells grown in the presence of 0.1 to 0.3 M NaCl, (iii) the growth rate decreased significantly with the addition of 0.5 M NaCl, and (iv) cell growth was drastically reduced in the presence of 0.6 M NaCl. Based on these results, MR-1 cells required NaCl levels between 0.1 to 0.3 M for optimal growth (5.8 to 17.5 g/liter) in aerobic MR2A medium. A slight decrease in overall growth was observed at 0.4 M NaCl; 0.5 M NaCl (29.2 g/liter) reduced the maximum growth rate twofold compared to the maximum growth rate observed at 0.1 to 0.3 M NaCl, and the maximum growth rate at 0.6 M NaCl was reduced over fourfold. For the present study, 0.5 M NaCl was used as a moderate stress for MR-1 cells.

Microarray analysis of salt adaptation response in MR-1. A whole-genome cDNA microarray was constructed and described previously (7, 13, 49). Briefly, gene-specific DNA fragments (<75% homology) were selected as probes with the software PRIMEGENS (52), and the primers were designed to amplify the gene-specific DNA fragments. A total of 4,648 pairs of gene-specific primers were designed based on the known sequences (13, 16) and synthesized. Gene-specific fragments were PCR amplified in 96-well plates 8 to 16 times in 100- μ l reaction mixtures, purified, pooled, quantified, and diluted to a minimum concentration of 50 ng/ μ l. Microarray fabrication, hybridization, and scanning were carried out as described previously (7, 13, 23, 49).

We harvested cells grown in the presence of 0.1 M (control condition) or 0.5 M (salt stress condition) NaCl for analysis. To evaluate biological variations, we extracted total cellular RNA from three sets of independent salt-stressed and control cultures to serve as biological replicates and that were hybridized at least twice for each replicate set by an optimized protocol (7, 13, 23, 49). The ratios of the salt-stressed samples to the con-

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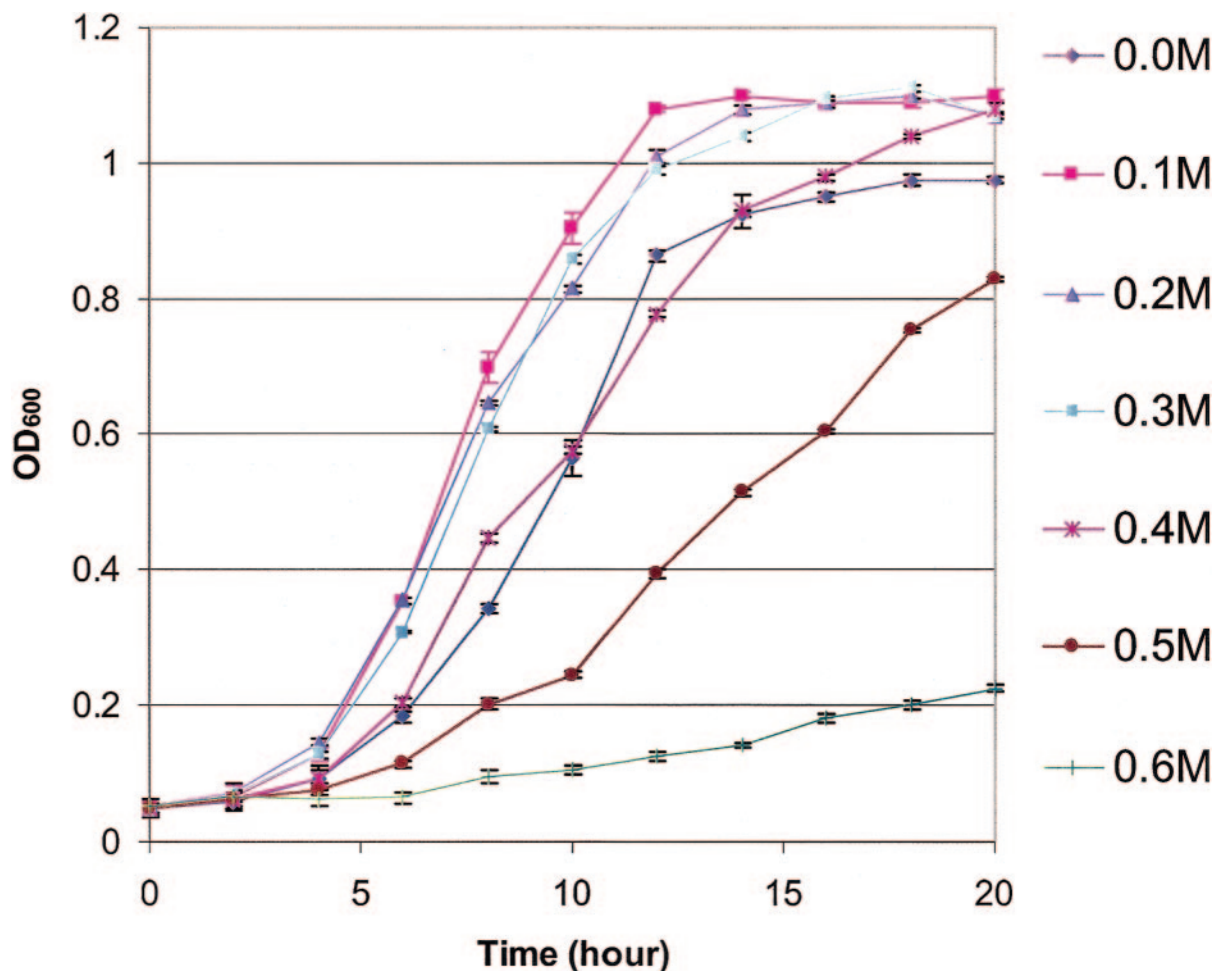


FIG. 1. Relationship between maximum growth rate of MR-1 cells grown in aerobic MR2A medium and increasing levels of sodium chloride.

trol samples for an arrayed gene were normalized by a trimmed geometric mean (48). Data points that were not consistently reproducible and had a disproportionately large effect on the statistical result were removed (23). Student's *t* test was used to identify differentially expressed genes by comparing the means of the normalized and log-transformed control versus salt-treated data with a total of 12 replicates in each set. A significance cutoff for the *t* statistic ($P = 0.05$) of a two-tailed test was chosen, and also required genes with significant changes to show a greater than twofold average change in expression level. As a result, a balance between the number of false negatives and trends supported by concerted changes among multiple genes within the same operon or pathway is achieved. For comparison, we also used the empirical Bayesian method of Lonnstedt and Speed (24) to rank and identify genes with significant changes, and the results are consistent by both methodologies.

The quality of the microarray data was assessed based on a number of criteria. First, expression patterns for genes in the same putative operons were checked. The similarity in gene expression patterns between gene pairs predicted to be in the same operon to that of randomly chosen gene pairs was compared. Consistent with this expectation, we observed that genes

within the same operon responded in a similar fashion under salt stress compared to genes randomly selected from the genome. Observed pairwise differences in log ratio expression levels were significantly smaller for the within-operon set (Kolmogorov-Smirnoff test, $D = 0.3925$, $P = < 2.2 \times 10^{-16}$) (37). Second, genes known to function together displayed similar changes in expression levels, as described throughout this article. One example is the consistent down-regulation of flagellar assembly genes (Table 1). Third, expression patterns of well-studied genes were verified (e.g., cation efflux transporters and Na^+/H^+ antiporters; Table 1S, online supplementary data [http://www.esd.ornl.gov/facilities/genomics/pubs/Table1S.xls]). Finally, we selected four predicted open reading frames (ORFs) that displayed significant changes in expression that have not been previously described as osmotic stress response genes in other organisms for real-time quantitative reverse transcription-PCR analysis (23). The expression patterns of the selected genes (*pflA*, *aceA*, *acnA*, and SO3874) were similar to the patterns observed with the microarrays (Table 2S, online supplementary material [http://www.esd.ornl.gov/facilities/genomics/pubs/Table2S.pdf]).

Overall genomic expression profile of MR-1 in response to salt stress. The overall genomic expression profiles indicated that the expression of a considerable subset of genes was af-

TABLE 1. Operon organizations and expression ratios of flagellar assembly genes in regions 1, 4, 5, and 6 and chemotaxis genes in regions 2 and 3

Gene ID no. ^a	Name	Putative function	Expression ratio ^b			Gene ID no.	Name	Putative function	Expression ratio		
			Avg	SD	Sig.				Avg	SD	Sig.
Region 1											
SO1529(-)	<i>motA-1</i>	Chemotaxis protein	0.470	0.184	Yes	SO3214		Hypothetical protein	0.369	0.154	Yes
SO1530(-)	<i>motB-1</i>	Chemotaxis protein	0.421	0.109	Yes	SO3215(+)	<i>flhB</i>	Flagellar biosynthetic protein	0.678	0.237	Yes
Region 2											
SO2120(-)	<i>cheY-1</i>	Chemotaxis protein	2.899	1.570	No	SO3216(+)	<i>flhR</i>	Flagellar biosynthetic protein	0.439	0.159	Yes
SO2121(-)	<i>cheA-1</i>	Chemotaxis protein	0.659	0.216	Yes	SO3217(+)	<i>flhQ</i>	Flagellar biosynthetic protein	0.672	0.252	No
SO2122(-)	<i>cheW-1</i>	Purine-binding chemotaxis protein	0.407	0.222	Yes	SO3218(+)	<i>flhP</i>	Flagellar biosynthetic protein	0.537	0.140	Yes
SO2123(-)		Methyl-accepting chemotaxis protein	1.004	0.390	No	SO3219(+)	<i>flhO</i>	Flagellar protein	0.532	0.210	Yes
SO2124(-)	<i>cheR-1</i>	Chemotaxis protein methyltransferase	0.934	0.387	No	SO3220(+)	<i>flhN</i>	Flagellar motor switch protein	0.575	0.138	Yes
SO2125(-)	<i>cheD-1</i>	Chemotaxis protein	0.865	0.293	No	SO3221(+)	<i>flhM</i>	Flagellar motor switch protein	0.716	0.363	No
SO2126(-)	<i>cheB-1</i>	Protein-glutamate methyltransferase	0.855	0.347	No	SO3222(+)	<i>flhL</i>	Flagellar protein	0.741	0.156	Yes
Region 3											
SO2317(-)		Methyl-accepting chemotaxis protein	1.295	0.590	No	SO3223(+)	<i>flhK</i>	Flagellar hook-length control protein	0.378	0.244	Yes
SO2318(-)	<i>cheY-2</i>	Chemotaxis protein	2.085	1.680	No	SO3224(+)	<i>flhJ</i>	Flagellar protein	0.328	0.168	Yes
SO2319		Anti-anti-sigma factor, putative	1.600	1.127	No	SO3225(+)	<i>flhI</i>	Flagellum-specific ATP synthase	0.577	0.161	Yes
SO2320(-)	<i>cheA-2</i>	Chemotaxis protein, interruption-N	1.600	1.127	No	SO3226(+)	<i>flhH</i>	Flagellar assembly protein	1.017	0.868	No
SO2321(-)		ISSod4, transposase	1.414	1.222	No	SO3227(+)	<i>flhG</i>	Flagellar motor switch protein	0.839	0.401	No
SO2322(-)	<i>cheA-2</i>	Chemotaxis protein, interruption-C	No data			SO3228(+)	<i>flhF</i>	Flagellar M-ring protein	0.584	0.132	Yes
SO2323(-)		Methyl-accepting chemotaxis protein	3.299	1.611	No	SO3229(+)	<i>flhE</i>	Flagellar hook-basal body complex	0.284	0.127	Yes
SO2324(-)	<i>cheW-2</i>	Purine-binding chemotaxis protein	1.933	1.425	No	SO3230(+)	<i>flhC</i>	Flagellar regulatory protein C	0.352	0.137	Yes
SO2325(-)	<i>cheR-2</i>	Chemotaxis protein methyltransferase	0.875	0.209	Yes	SO3231(+)	<i>flhB</i>	Flagellar regulatory protein B	0.200	0.073	Yes
SO2326(-)	<i>cheD-2</i>	Chemotaxis protein, putative	1.197	0.618	No	SO3232(+)	<i>flhA</i>	Flagellar regulatory protein A	0.388	0.150	Yes
SO2327(-)	<i>cheB-2</i>	Protein-glutamate methyltransferase	1.373	0.666	No	SO3233(+)	<i>flhS</i>	Flagellar protein	0.527	0.183	Yes
Region 4											
SO3202(-)	<i>cheW-3</i>	Purine-binding chemotaxis protein	1.247	0.495	No	SO3235(+)	<i>flhD</i>	Hypothetical protein	0.497	0.273	Yes
SO3203(-)		CheW domain protein	0.987	0.300	No	SO3236(+)	<i>flhG</i>	Flagellar hook-associated protein	0.555	0.304	Yes
SO3204		ParA family protein	1.103	0.368	No	SO3237(+)		Flagellin	0.345	0.078	Yes
SO3205		Hypothetical protein	1.233	0.398	No	SO3238(+)		Flagellin	0.317	0.097	Yes
SO3206(-)	<i>cheB-3</i>	Protein-glutamate methyltransferase	1.615	0.717	No	SO3239(+)		Flagellin	0.846	0.471	No
SO3207(+)	<i>cheZ</i>	Chemotaxis protein	1.226	0.387	No	SO3241(+)	<i>flgL</i>	Flagellar hook-associated protein	0.495	0.226	Yes
SO3208(+)	<i>cheY-3</i>	Chemotaxis protein	1.722	0.908	No	SO3242(+)	<i>flgI</i>	Flagellar protein	0.422	0.216	Yes
SO3209(+)	<i>flh4</i>	RNA polymerase sigma-28 factor	1.886	0.940	No	SO3243(+)	<i>flgH</i>	Flagellar P-ring protein	0.383	0.229	Yes
SO3210(+)	<i>flhG</i>	Flagellar biosynthetic protein	1.052	0.331	No	SO3244(+)	<i>flgG</i>	Flagellar L-ring protein	0.427	0.170	Yes
SO3211(+)	<i>flhF</i>	Flagellar biosynthetic protein	0.749	0.141	No	SO3245(+)	<i>flgF</i>	Flagellar basal-body rod protein	0.389	0.175	Yes
SO3212(+)		Flagellar biosynthesis protein	0.632	0.293	Yes	SO3246		Flagellar basal-body rod protein	0.320	0.188	Yes
SO3213(+)	<i>flh4</i>	Flagellar biosynthesis protein	0.558	0.149	Yes	SO3247(+)	<i>flgE</i>	Hypothetical protein	0.434	0.143	No
Region 5											
SO3936(+)	<i>motX</i>	Sodium-type flagellar protein	0.788	0.323	No	SO3248(+)	<i>flgD</i>	Hypothetical protein	0.269	0.136	Yes
Region 6											
SO4286(+)	<i>motB-2</i>	Chemotaxis <i>motB</i> protein	0.737	0.328	No	SO3249(+)	<i>flgC</i>	Basal-body rod modification protein	0.276	0.066	Yes
SO4287(+)	<i>motA-2</i>	Chemotaxis <i>motA</i> protein	0.646	0.135	Yes	SO3250(+)	<i>flgB</i>	Flagellar basal-body rod protein	0.300	0.109	Yes
						SO3251(+)	<i>cheR-3</i>	Chemotaxis protein methyltransferase	0.142	0.053	Yes
						SO3252(+)	<i>cheV</i>	Chemotaxis protein	0.609	0.188	Yes
						SO3253(-)		Chemotaxis protein	0.556	0.113	Yes
						SO3254(-)	<i>flgM</i>	Flagellar basal-body P-ring protein	0.257	0.071	Yes
						SO3255(-)	<i>flgN</i>	Regulator of flagellin synthesis	0.307	0.164	Yes
								Flagellar biosynthetic protein	0.190	0.086	Yes

^a + and - represent the operon location on the positive or negative strand, respectively. Some non-motility-related genes are also included in the table to keep the entry of a complete operon.

^b The average expression ratio of the salted sample to the control was calculated from 12 replicates together with the respective standard deviation (SD), a $P = 0.05$ standard t test result (significant [Sig.]).

ected during growth in the presence of 0.5 M NaCl. We identified a total of 518 genes (11.2% of the total gene content) as significantly upregulated and 598 genes (13%) as significantly down-regulated by a factor of 2 or more. According to the genome sequence annotations provided by The Institute for Genomic Research (<http://www.tigr.org/>), the majority of the up-regulated genes fell into the following functional categories: amino acid biosynthesis, protein synthesis, biosynthesis of cofactors, prosthetic groups, energy metabolism, and fatty acid/phospholipid metabolism. A large fraction of the most-highly-down-regulated genes were annotated as chemotaxis-related proteins. Similar to previous studies of microbial stress response (19, 23, 42, 53), changes in the expression of ORFs predicted to be involved with protein biosynthesis seem to play an important role in modulating cellular activities that allow adaptation to environmental stress (Table 1S).

Salt stress activated genes involved in Na⁺ efflux and K⁺ accumulation. Na⁺ extrusion and replacement with K⁺ is the primary response of *Escherichia coli* to NaCl stress. To balance the large amounts of cation accumulation, *E. coli* will also accumulate glutamate (46). MR-1 appears to respond similarly to NaCl stress. First, genes encoding K⁺ uptake proteins were up-regulated, as well as Na⁺ efflux system components that included the Trk K⁺ uptake system, Na⁺/H⁺ antiporters, and Na⁺ efflux transporters (Table 1S). As expected, genes (SO1325 and SO4410) putatively involved in glutamate synthesis and a Na⁺/glutamate symporter gene (SO2923) were up-regulated in MR-1 by NaCl stress (Table 1S).

Besides the primary response, a secondary response (i.e., the accumulation of compatible osmolytes) may occur when a cell is subjected to salt concentrations of 0.5 M or higher, as observed in *E. coli* (46). Genes that encode the enzymes for trehalose and estoine biosynthesis, however, have not been identified in MR-1, and the corresponding compounds have not been reported. Sequence annotation of the MR-1 genome revealed two operons that contain *proABC* genes encoding enzymes for proline synthesis (SO1121, SO1122, and SO3354), but the expression of these genes was not significantly changed under the salt stress conditions examined (Table 1S). Interestingly, the accumulation of glycine betaine was observed in *S. oneidensis* cells grown in the presence of salted and smoked salmon (20). The authors stated that exogenous choline in the fish was transported and converted to glycine betaine (20). Therefore, MR-1 appears to have the ability to synthesize glycine betaine from choline. Generally, choline is first oxidized to glycine betaine aldehyde by the enzyme choline dehydrogenase (BetA) in *E. coli* or by a type III alcohol dehydrogenase (GbsB) in *Bacillus subtilis*. The intermediate glycine betaine aldehyde is then further oxidized to glycine betaine by glycine betaine aldehyde dehydrogenase BetB in *E. coli* or GbsA in *B. subtilis* (46). We identified two candidates (SO3496 and SO4480) for aldehyde dehydrogenase, one gene for type II (SO1490) and one gene for type III alcohol dehydrogenase (SO2054), but no candidates for choline dehydrogenase. These candidates, however, may function together to convert choline into glycine betaine in MR-1. The two putative alcohol dehydrogenase genes (SO3498 and SO4480) were slightly but not significantly up-regulated, and the other two aldehyde dehydrogenase genes (SO1490 and SO2054) were significantly

down-regulated. It is therefore unlikely that glycine betaine biosynthesis was enhanced under the growth conditions tested.

Up-regulation of respiration-related genes. Microarray analyses indicated that genes involved in both aerobic and anaerobic respiration were significantly up-regulated in salt-stressed MR-1 cells (Fig. 2 and Table 1S). The up-regulated genes involved in aerobic respiration included tricarboxylic acid (TCA) cycle enzymes and ATP synthase (SO4746 to SO4753), and ORFs predicted to play a role in anaerobic respiration included components of fumarate, nitrate, and nitrite reductases. Consistent with the activation of these enzymes, key genes involved in the biosynthesis of such cofactors as molybdopterin (2, 17, 50), heme (55), and menaquinone (31, 41) were also up-regulated (Table 1S). Up-regulated genes reported to be involved in fermentation were also observed, including formate dehydrogenase, quinone-reactive Ni/Fe hydrogenase, and acetate kinase.

Pyruvate can be respired either aerobically through the TCA cycle or anaerobically by formate dehydrogenation and fermentation. The pyruvate formate-lyase encoded by *pflAB* is the key enzyme that catalyzes pyruvate to formate (1), leading to the final products H₂ and CO₂. Significant up-regulation of the *pflAB* genes was observed, suggesting a possible redirection of pyruvate. At the same time, an operon that contained aconitase, methylcitrate synthase, methylisocitrate lyase, and a conserved hypothetical protein was up-regulated 6.7- to 9.1-fold. The apparent up-regulation of both aerobic and anaerobic respiration genes has also been reported for *E. coli* cells exposed to seawater for 20 h (40).

The glyoxylate bypass can reduce NADH production as well as allow a partial TCA to function to generate intermediates for anabolic reactions (e.g., amino acid biosynthesis) without the decarboxylation steps that result in loss of carbon (CO₂). The methylcitric acid pathway can provide additional energy from fatty acid and acetate catabolism. Apparently, the cell needs energy to survive the stress, but the aerobic respiration that can produce more energy may simultaneously generate extra reactive oxygen species as by-products (43), thus resulting in oxidative stress. This effect was observed in the moderately halophilic *Shewanella* sp. strain CN32, which requires 5 to 6% NaCl for optimal growth (4). Up-regulation of anaerobic respiration could help reduce oxidative stress to the cell. In addition, the cells may undergo clumping as a protective response to osmotic stress, as observed in *Azospirillum brasilense* (18) and *Vibrio cholerae* (51), and therefore may experience microaerophilic or anoxic conditions. However, the aggregation of MR-1 cells during salt stress was investigated as previously described (18), and significant aggregation was not detected for either the control or salt-stressed cells (data not shown). Observation of the cells by light microscopy also supported this conclusion. However, the cells were shaken during incubation, and significant clumping might have been prevented. Further work is needed to discern the possible connection between clumping and anaerobic metabolism.

Down-regulation of flagellar assembly genes impacted cell motility. Phylogenetic analysis suggested that *S. oneidensis* flagellar motor proteins were more closely related to the sodium-driven motors in *Vibrio* species than to proton-driven motors. In addition, homologs of the MotAB and MotXY proteins, which are thought to be associated with sodium-driven

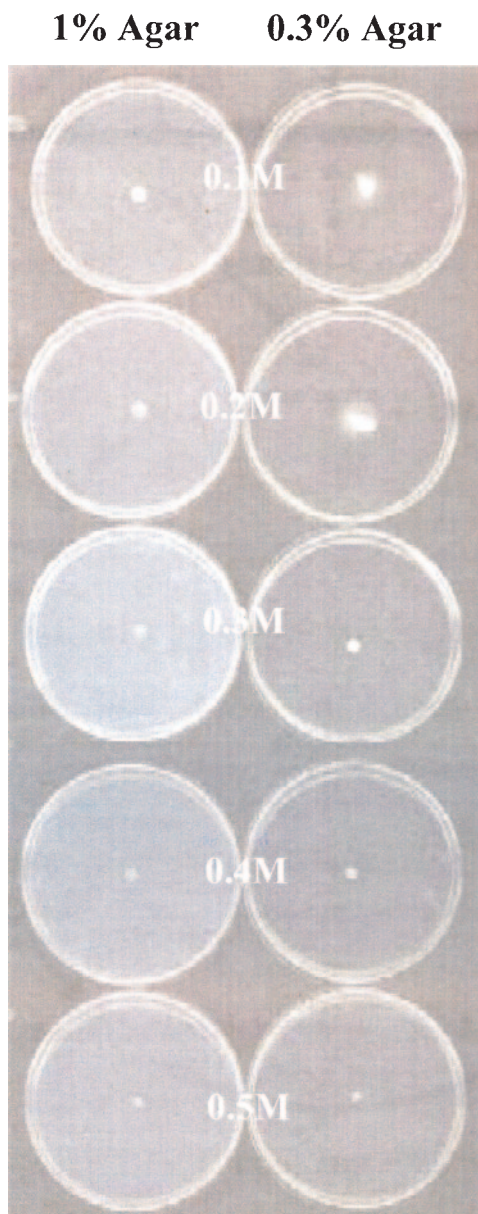


FIG. 3. NaCl at a concentration of 0.3 M or higher completely halts MR-1 cell motility. The solid medium plates (1% agar) are designed to control possible different cell growth rates over varied NaCl concentrations as indicated, whereas the semisolid medium plates (0.3% agar) show the cell ability for motility.

a sizable investment with respect to cellular economy. In *V. cholerae*, the operons constitute a large, coordinately regulated flagellar regulon that is divided into three temporally regulated, hierarchical transcriptional levels: early, middle, and late (27). In *V. cholerae*, FlrA, acting as a σ^{54} -dependent transcription factor, activates transcription of *flrBC*, a two-component signal transduction system. The phosphorylation of FlrC by FlrB is required to activate middle-level flagellar genes (38), which includes most flagellar assembly genes, and *fliA*, which encodes a specialized sigma factor, σ^{28} . σ^{28} activity controls transcription of the late-level genes like the flagellin, motor, and anti-sigma factor genes (27). Salt stress repressed the expression of

flrA and *flrC*, the master transcriptional regulator genes in MR-1, leading to a complete shutdown of middle- and late-level flagellar assembly genes (Table 1). MR-1 may be similar to *E. coli* in terms of flagellar gene expression regulation, in which a promoter or promoters of the master operon *fliDC* receive a number of global regulatory signals, including the concentration of inorganic salt (9). The simultaneous detection of the whole-genomic expression patterns in response to a specific environmental stress can provide details about the possible connections between components in regulatory networks.

Concluding remarks. The up-regulation of energy metabolism, including electron transport, and down-regulation of flagellar biosynthesis in response to elevated salt conditions suggested that MR-1 needs more ATP to pump sodium out of the cell. In addition, an increase in electron transport may directly contribute to the efflux of sodium via the sodium-translocating electron transport complex I. Under high-salt conditions, MR-1 may repress the expression of flagellar genes to conserve energy necessary for sodium transport. The genomic expression profile of MR-1 in response to the sodium salt stress together with comparative genomics analyses indicated that MR-1 resembled responses observed in *V. cholerae*. As with *Vibrio* (10), a majority of *Shewanella* species reside in oceans, coastal waters, and estuaries and were therefore more tolerant to sodium salt stress. More genomic similarities of MR-1 to *V. cholerae* clearly outline the connections between environments where the microorganisms naturally reside.

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