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Global Transcriptional, Physiological, and Metabolite Analyses of the Responses of *Desulfovibrio vulgaris* Hildenborough to Salt Adaptation▽†

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The response of *Desulfovibrio vulgaris* Hildenborough to salt adaptation (long-term NaCl exposure) was examined by performing physiological, global transcriptional, and metabolite analyses. Salt adaptation was reflected by increased expression of genes involved in amino acid biosynthesis and transport, electron transfer, hydrogen oxidation, and general stress responses (e.g., heat shock proteins, phage shock proteins, and oxidative stress response proteins). The expression of genes involved in carbon metabolism, cell growth, and phage structures was decreased. Transcriptome profiles of *D. vulgaris* responses to salt adaptation were compared with transcriptome profiles of *D. vulgaris* responses to salt shock (short-term NaCl exposure). Metabolite assays showed that glutamate and alanine accumulated under salt adaptation conditions, suggesting that these amino acids may be used as osmoprotectants in *D. vulgaris*. Addition of amino acids (glutamate, alanine, and tryptophan) or yeast extract to the growth medium relieved salt-related growth inhibition. A conceptual model that links the observed results to currently available knowledge is proposed to increase our understanding of the mechanisms of *D. vulgaris* adaptation to elevated NaCl levels.

Desulfovibrio vulgaris Hildenborough is a member of the anaerobic sulfate-reducing bacteria (SRB) (46) that are ubiquitous in anaerobic environments containing sulfate (43), such as gas pipelines, subsurface metal tanks, sediments, and offshore oil production facilities (6, 63). These niches can also contain high concentrations of salt (e.g., NaCl). Because of its capacity to immobilize soluble forms of toxic metals, the potential of *D. vulgaris* for bioremediation has been widely recognized (35, 57). In addition, *Desulfovibrio* species have been found to reduce metals in sediments and soils with high concentrations of NaCl and a mixture of toxic metals (3) and to cope with salt stresses that result from environmental hydration-dehydration cycles. Therefore, further understanding of mechanisms that *D. vulgaris* uses to survive and adapt to environments with high concentrations of salt (e.g., NaCl) may contribute to the development of successful bioremediation strategies. Such knowledge might also be useful for prediction and control of metal biocorrosion given the apparent role of this microbe in corrosion.

The response of a microorganism to high salinity can be divided into two phases. The initial reaction to a sudden increase in the salt concentration is termed "salt shock," while the subsequent survival and growth in a high-salinity environment can be termed "salt adaptation." In both cases, exposure of *D. vulgaris* to high salinity may present two different, but related, environmental stimuli; one of these stimuli is osmotic stress, and the other is ionic stress (19). Hyperosmotic stress triggers water efflux from the cell that results in a reduction in the turgor pressure and dehydration of the cytoplasm, which increases the ion concentration in the cytosol, whereas ionic stress causes ions (e.g., Na^+) to enter the cytoplasm, which leads to a further increase in the ion concentration and subsequently damages the membrane systems and deactivates key enzymes (50).

Bacteria use a range of mechanisms to respond to high salinity. The most common mechanism is accumulation of compatible solutes, such as glutamate (8, 9, 18, 38, 41), trehalose (55), proline (61), glycine betaine (32), or ectoine (29). These solutes increase the internal osmotic pressure without interfering with vital cellular protein functions (15) and are accumulated either by biosynthesis or by import. Another strategy used by bacteria is exclusion of harmful ions (e.g.,

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Na^+) via a variety of transport systems, such as Na^+/H^+ antiporters and Ca^{2+}/Na^+ exchangers (34, 36, 44), which may be present constitutively or induced in the presence of high salinity. Na^+/H^+ antiporters also play key roles in salt tolerance in cyanobacteria (59) and *Arabidopsis* (49). The bacterial responses to salt stress also include induction of chaperones, chaperone-like proteins, and peptidases to eliminate incorrectly folded macromolecular structures caused by high salinity (22).

Genome-wide transcriptional analyses of salt and/or osmotic stress responses have been performed for many different bacteria and archaea, including *Escherichia coli* (60), *Bacillus subtilis* (52), *Shewanella oneidensis* MR-1 (34), *Yersinia pestis* (19), *Pseudomonas aeruginosa* (1), *Sinorhizobium meliloti* (16), and *Methanosarcina mazei* (45, 51). A broad set of differentially expressed genes was observed in these studies. Since the annotated *D. vulgaris* genome is available (24), a series of transcriptional studies of *D. vulgaris* responses to different environmental stresses have been conducted (4, 13, 14, 20, 40, 54). In particular, a study of the response of *D. vulgaris* to salt shock demonstrated that import of osmoprotectants (e.g., glycine betaine and ectoine) was the primary mechanism used to counter hyperionic stress (39). This study also showed that several efflux systems, ATP synthesis pathways, and chemotaxis genes were highly upregulated, while flagellar biosynthesis and lactate uptake and transport systems were downregulated (39). In other organisms, such as *B. subtilis*, both salt shock and salt adaptation have been studied. Only a small number of differentially expressed genes were found for both treatments (52). Thus, it was of interest to explore the adaptive response of *D. vulgaris* by comparing it with our previous characterization of salt shock (39) and examining its relationship to the adaptive physiology of other bacteria.

In this study, possible mechanisms of *D. vulgaris* adaptation to high salinity were explored using transcriptome profiling combined with cell growth, physiological, and metabolite analyses. Here, salt adaptation was defined as growth of *D. vulgaris* with high levels of salt for ca. 100 h, after which the cells are still in an apparently exponential growth phase. In contrast, salt shock was defined operationally as exposure of *D. vulgaris* cells to NaCl for a shorter time (30 to 480 min) during the exponential phase (39). Based on these working definitions, our objectives in this study were to (i) examine transcript changes, especially changes in amino acid metabolism and transport, as well as energy metabolism, in *D. vulgaris* in response to salt adaptation; (ii) identify potential osmoprotectants in *D. vulgaris* during salt adaptation; (iii) compare the global transcriptome profile of *D. vulgaris* in response to salt adaptation with the profile in response to salt shock; and (iv) elucidate physiological states for initial and prolonged responses to NaCl stress. Based on these studies, we propose a conceptual model for *D. vulgaris* adaptation to high salinity.

MATERIALS AND METHODS

Oligonucleotide probe design and microarray construction. A 70-mer oligonucleotide microarray for *D. vulgaris* was designed and constructed as described previously (20). All oligonucleotides designed were commercially synthesized without modification by MWG Biotech Inc. (High Point, NC). The concentration of oligonucleotides was adjusted to 100 pmol/μl. Oligonucleotide probes prepared in 50% dimethyl sulfoxide (Sigma Chemical Co., MO) were spotted onto UltraGAPS glass slides (Corning Life Science, NY) using a Microgrid II arrayer (Genomic Solutions Inc., MI). For each oligonucleotide probe there were two replicates on a single slide. Additionally, six concentrations (5 to 300 ng/μl) of genomic DNA were also spotted (4 replicates on a single slide) as general positive controls. In total, there were 7,284 spots on the array. After printing, the oligonucleotide probes were fixed onto the slides by UV cross-linking (600 mJ) according to the protocol of the manufacturer (Corning Life Science, NY).

Cell growth. *D. vulgaris* cultures were routinely grown in a defined lactate sulfate medium (LS4D medium) at 37°C. LS4D medium consisted of 60 mM sodium lactate, 50 mM Na_2SO_4, 8.0 mM $MgCl_2$, 20 mM NH_4Cl, 2.2 mM K_2HPO_4, 0.6 mM $CaCl_2$, 30 mM PIPES [piperazine-*N*,*N*-bis(2-ethanesulfonic acid)], 12.5 ml of a trace mineral solution per liter (10), NaOH (to adjust the pH to 7.2), and 1.0 ml of a 10× vitamin solution per liter (10) that was added after autoclaving. The reductant used for LS4D medium was 5 ml per liter of an anaerobic titanium citrate solution. This solution contained 20% (wt/vol) titanium(III) chloride, 0.2 M sodium citrate, and 8.0% (wt/vol) sodium carbonate. Cell growth was monitored using the optical density at 600 nm (OD_{600}). For transcript and metabolite analyses, control (without additional NaCl) and NaCl treatment (with 250 mM NaCl) samples were harvested at the mid-log phase (OD_{600}, ~0.25) approximately 15 and 100 h after inoculation, respectively, in triplicate at room temperature under anaerobic conditions, snap-frozen in liquid nitrogen, and then stored at −80°C. To determine the effects of amino acids on *D. vulgaris* growth, 100-μl portions of 200 mM amino acid solutions (final concentration, 2 mM) that were prepared and filter sterilized under anaerobic conditions were added to LS4D medium with or without 250 mM NaCl. Ten-milliliter cultures were incubated at 37°C in 30-ml anaerobic culture tubes closed with butyl rubber stoppers and aluminum seals.

RNA extraction, purification, and labeling. Total cellular RNA was isolated using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). RNA samples were treated with RNase-free DNase I (Ambion, Austin, TX) and were purified using an RNeasy mini kit (Qiagen, Chatsworth, CA). Ten micrograms of total cellular RNA was used for labeling as described previously (20). The labeled cDNA probes were purified immediately using a QIAquick PCR purification column and were concentrated using a Savant Speedvac centrifuge (Savant Instruments Inc., Holbrook, NY). For each biological sample, three slides were used. In addition to the duplicates of arrays on the same slide, three biological cell samples were used to produce a total of 18 possible spots for each gene.

Genomic DNA extraction, purification, and labeling. Genomic DNA was isolated and purified from *D. vulgaris* as described previously (65). The purified genomic DNA was fluorescently labeled by random priming using the Klenow fragment of DNA polymerase as described previously (21). The labeled genomic DNA was purified immediately using a QIAquick PCR purification column and was concentrated using a Savant Speedvac centrifuge (Savant Instruments Inc., Holbrook, NY).

Microarray hybridization, washing, and scanning. Labeled genomic DNA (Cy3) was used as a common reference for cohybridization with labeled RNA (Cy5) samples for each slide. Hybridization was performed using a TECAN HS4800 Pro hybridization station (TECAN U.S., Durham, NC) and the protocol recommended by the manufacturer. This system allows hybridization, washing, and drying of arrays automatically and provides more sensitive and consistent hybridization results. After hybridization, slides were scanned using a ProScanArray microarray analysis system (Perkin Elmer, Boston, MA).

Microarray data analysis. To determine fluorescent signal intensities for each spot, 16-bit TIFF scanned images were analyzed using ImaGene version 6.0 (Biodiscovery Inc., Los Angeles, CA) to determine the spot signal, spot quality, and background fluorescence intensities. Any flagged spots (e.g., empty spots and bad spots) were removed before normalization. Details of the microarray data analysis procedure were described previously by Mukhopadhyay et al. (39). Complete microarray data have been deposited in the NCBI GEO database under accession number GSE 14343.

Real-time PCR quantification. In order to validate microarray hybridization results, 25 genes were selected for further analysis with real-time PCR. A specific primer pair for each gene was designed to produce a 98- to 119-bp product, and detailed information about the genes selected and their primers is shown in Table S1 in the supplemental material. Real-time PCR (25 μl) was performed using Thermo-fast 96 PCR plates (Bio-Rad Laboratories, Hercules, CA), which were sealed with iCycler IQ optical-quality tape (Bio-Rad Laboratories), and an iCycler IQ thermocycler (Bio-Rad Laboratories). Each measurement was obtained for three replicates. A series of dilutions (10 to 10^9 copies) of the *recA* amplicon were used as calibration standards in each plate. First, *recA* was amplified (product size, 877 bp) using recAF1 (ATTGGAGACCGCTCTTAGCA) and recAR1 (CTGGCCCAGTCTCTCTGAAC). Second, the amplicon was

purified using a QIAquick PCR purification kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. Third, the purified PCR fragment was visualized, and its size was confirmed by agarose gel electrophoresis, using a PicoGreen double-stranded DNA assay kit (Invitrogen, Carlsbad, CA). Finally, the quantified amplicon was serially diluted to generate a standard curve for a product size of 114 bp using recAF2 (CGTCAACACCGAAGAGCTG) and recAR2 (ACGGAGTCGATGACCACAA). Data analysis was carried out with iCycler software (Bio-Rad Laboratories). Based on the standard curve, a cycle threshold (C_T) value was converted to the copy number of the gene in each sample. The ratio of the copy number for the treatment to the control copy number was calculated.

Metabolite extraction. Metabolites were extracted using a methanol-water-chloroform extraction procedure, after which solid-phase extraction (Oasis HLB, Waters, MA) was used to remove salts from the sample (2). All amino acids except betaine, glutamate, and serine were quantified using stable isotope-labeled amino acid standards, which were purchased from Sigma-Aldrich (MO) and C/D/N Isotopes (Quebec, Canada). Betaine, glutamate, and serine were quantified based on the percentages of recovery from the solid-phase extraction cartridge.

CE conditions and MS. The capillary electrophoresis (CE) separation conditions used were the conditions previously described by Baidoo et al. (2). However, for detection of betaine, glutamate, and serine, the sample was introduced into the capillary using a pressure of 50×10^2 Pa for 250 s. Mass spectrometry (MS) analysis was conducted using an Agilent 6210 time of flight liquid chromatograph-mass spectrometer (TOF LC/MS) (Agilent Technologies, Santa Clara, CA) and an Agilent series 1100 isocratic high-performance liquid chromatography (HPLC) pump for sheath liquid delivery. CE and electrospray ionization (ESI) MS coupling was achieved using an orthogonal coaxial sheath-flow interface, and the Agilent CE system was interfaced with the Agilent 6210 TOF LC/MS via a G1603A Agilent CE-MS adapter kit and a G1607A Agilent CE-ESI-MS sprayer kit (Agilent Technologies, Santa Clara, CA). Both the Agilent CE system and the Agilent 6210 TOF LC/MS were controlled by the Chemstation software package (Agilent Technologies, Santa Clara, CA). A contact closure between the two instrument setups was established in order to trigger the MS to operate upon initiation of a Chemstation run cycle. The Agilent 6210 TOF LC/MS was initially calibrated using the ES tune mixture (Agilent Technologies, Santa Clara, CA) and was internally calibrated during runs using reference masses from tetrabutylammonium acetate (Sigma-Aldrich, St. Louis, MO) and tetraethylammonium acetate (Fluka, Seelze, Germany). Grounding of the CE-ESI-MS sprayer ensured that a full 30-kV potential difference was applied across the length of the capillary for more efficient separation. An electrical contact at the outlet end was provided by the sheath liquid (methanol-water, 50:50 [vol/vol]) at a flow rate of 8 μl/min. Nitrogen gas was used as both the nebulizing gas (8 lb/in^2) and the drying gas (8 liters/min) to facilitate the production of gas-phase ions. A drying gas temperature of 200°C was used throughout the study. Data acquisition and processing were carried out using the Agilent MassHunter Work Station Console software package.

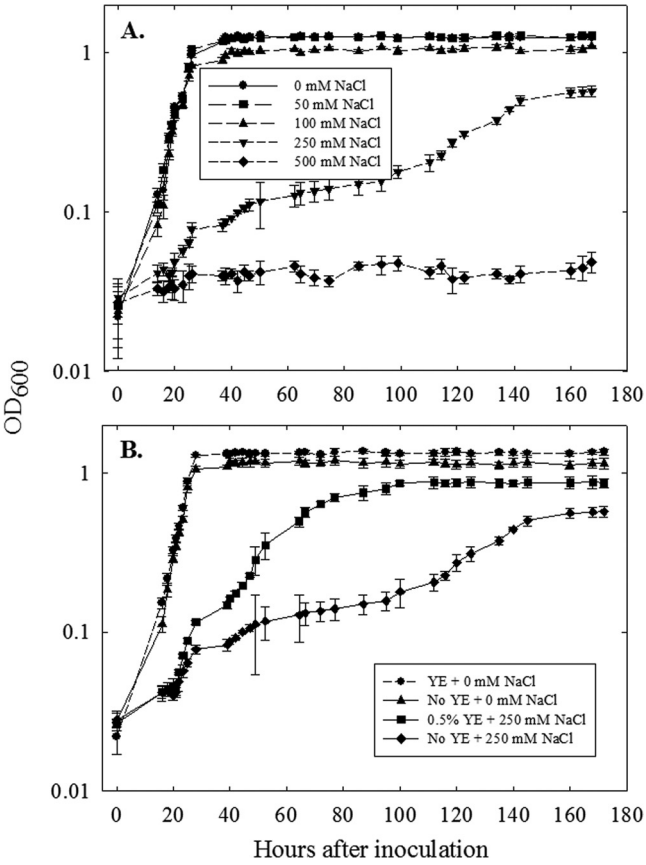

FIG. 1. Growth curves for *D. vulgaris* grown in LS4D medium. The stock culture was inoculated into LS4D medium and incubated overnight before use in the experiment. (A) An overnight culture was inoculated (1.0%) into LS4D medium containing 0, 50, 100, 250, or 500 mM NaCl. (B) An overnight culture was inoculated (1.0%) into LS4D medium containing 250 mM NaCl with or without 0.5% (wt/vol) yeast extract (YE), and controls without NaCl or without yeast extract were also included.

RESULTS

Growth of *D. vulgaris* during salt adaptation. *D. vulgaris* cells were grown in LS4D medium with different concentrations of NaCl (0 to 500 mM) (Fig. 1A). Low NaCl concentrations (up to 100 mM) did not significantly affect cell growth, as monitored by the OD_{600}. The maximal OD_{600} was adversely affected by 250 mM NaCl; it was decreased by 50% (Fig. 1A). *D. vulgaris* could not grow in LS4D medium with 500 mM NaCl (Fig. 1A). To determine the effects of yeast extract on the growth of *D. vulgaris* with a high level of salt, cells were grown in LS4D medium with or without 0.5% (wt/vol) yeast extract. After addition of 250 mM NaCl, the culture with yeast extract had an OD_{600} of approximately 0.9, compared with an OD_{600} of 0.6 for the culture without yeast extract, and yeast extract allowed more rapid growth, although both the amount and the rate of growth were less than the amount and the rate of growth in the control culture not challenged with a high level of salt (Fig. 1B). The results indicated that yeast extract was beneficial for *D. vulgaris* growth and that some of its components (e.g., amino acids) could mitigate growth defects during adaptation of *D. vulgaris* to NaCl, and this has been observed previously (17). All other experiments to examine the transcriptional, metabolic, and physiological responses of *D. vulgaris* were conducted using 250 mM NaCl.

Transcriptional analysis of *D. vulgaris* responses to salt adaptation. To assess transcript changes in cultures of *D. vulgaris* grown for an extended time with a high level of salt, the Z score cutoff for significant changes (|Z|) was set at ≥2.0. Using this criterion, of 2,647 of the 3,634 open reading frames (ORFs) that were assigned to 20 functional categories based on The Institute for Genome Research (TIGR) functional roles, 195 and 184 were significantly upregulated and downregulated, respectively (see Fig. S1 in the supplemental material). Almost 30% of the genes with significantly altered expression encode hypothetical proteins, conserved hypothetical proteins, and proteins with unknown functions (111 ORFs or 15.2% of the genes assigned to these categories). The other categories of significantly changed (up- or downregulated) genes included genes involved in amino acid metabolism (8 ORFs [10.3% of the genes assigned to this category]), protein synthesis and fate

TABLE 1. Changes in expression of *D. vulgaris* genes involved in amino acid biosynthesis and transport

Locus	Gene	Predicted function	Salt adaptation conditions		Salt shock conditions	
			$Log_2 R$ (treatment/control)	Z score	$Log_2 R$ (treatment/control)	Z score
Amino acid metabolism						
DVU0085	*trpB-1*	Tryptophan synthase, beta subunit	2.48	3.72	1.44	2.74
DVU0470	*trpB-2*	Tryptophan synthase, beta subunit	−0.01	−0.01	1.16	2.17
DVU0471	*trpA*	Tryptophan synthase, alpha subunit	0.09	0.15	1.41	2.73
DVU2204	*tnaA*	Tryptophanase	1.81	3.22	0.27	0.47
DVU0115	*aroE*	Shikimate 5-dehydrogenase	1.58	2.01	0.74	1.19
DVU0375	NA[a]	Glu/Leu/Phe/Val dehydrogenase family protein	2.70	3.69	0.70	1.12
DVU1823	*gltB-1*	Glutamate synthase, iron-sulfur clustering-binding subunit, putative	0.20	0.16	1.35	2.37
DVU3291	NA	Glutamate synthase, iron-sulfur clustering-binding subunit, putative	0.30	0.48	1.54	2.31
DVU2981	*leuA*	2-Isopropylmalate synthase	−0.18	−0.18	1.36	2.51
DVU2982	*leuC*	3-Isopropylmalate dehydratase, large subunit, putative	−1.62	−1.67	1.23	1.94
DVU2983	*leuD*	3-Isopropylmalate dehydratase, small subunit	−2.37	−2.23	1.01	1.86
DVU2984	NA	Conserved hypothetical protein	−3.14	−3.46	0.98	1.87
DVU2985	*leuB*	3-Isopropylmalate dehydrogenase	−1.54	−2.25	0.68	1.35
Amino acid transport						
DVU0724	NA	Sodium/Ala symporter family protein	1.42	2.69	0.53	1.04
DVU2297	*proW*	Glycine/betaine/L-proline ABC transporter, periplasmic-binding protein	1.59	3.11	1.62	3.13
DVU2298	*opuBB*	Glycine/betaine/L-proline ABC transporter, permease protein	0.82	1.42	1.43	2.74
DVU2299	*proV*	Glycine/betaine/L-proline ABC transporter, ATP binding protein	0.87	1.49	1.48	2.56
DVU2341	NA	Amino acid ABC transporter, permease protein, His/Glu/Gln/Arg/opine family	1.56	2.76	0.78	1.18
DVU2740	*livF*	High-affinity branched-chain amino acid ABC transporter, ATP-binding protein	3.18	5.34	0.61	0.96
DVU2741	*livG*	High-affinity branched-chain amino acid ABC transporter, ATP-binding protein	0.54	0.64	1.05	1.66
DVU2742	*livM*	High-affinity branched-chain amino acid ABC transporter, permease protein	1.79	2.08	0.93	1.76
DVU2743	*livH*	High-affinity branched-chain amino acid ABC transporter, permease protein	0.29	0.32	0.15	0.22
DVU2744	NA	High-affinity branched-chain amino acid ABC transporter, periplasmic amino acid binding protein	2.40	4.48	1.78	3.20
DVU3297	*mtr*	Tryptophan-specific transport protein	1.34	2.48	0.35	0.65

[a] NA, no gene name has been assigned to the locus yet.

(16 ORFs [6.3%]), DNA metabolism (8 ORFs [9.6%]), cell envelope synthesis (32 ORFs [13.0%]), cellular processes (22 ORFs [13.8%]), energy metabolism (37 ORFs [14.2%]), mobile and extrachromosomal element functions (39 ORFs [30.5%]), signal transduction and regulatory functions (37 ORFs [14.2%]), and transport and binding proteins (41 ORFs [16.5%]) (see Fig. S1 in the supplemental material). In this study, our analysis focused on the genes involved in amino acid metabolism and transport, energy metabolism, regulatory functions, and general stress responses.

(i) Amino acid metabolism and transport. The expression of 49 genes of the 226 ORFs in the amino acid metabolism and transport category was significantly changed under salt adaptation conditions, and the genes with the largest changes are shown in Table 1. *DVU0375*, encoding a putative Glu/Leu/Phe/Val dehydrogenase family protein, was expressed at a high level when *D. vulgaris* was adapted to 250 mM NaCl. The increase in expression might reasonably be expected to result in an increase in the biosynthesis of glutamate, leucine, phenylalanine, and/or valine. *aroE*, which encodes a shikimate 5-hydrogenase that produces shikimate, a precursor of aromatic amino acids, was also upregulated, as were *trpB-1*, which encodes the beta subunit of a tryptophan synthase, and *tnaA*, which encodes a tryptophanase. The *D. vulgaris* genome has a predicted five-gene operon (*DVU2981* to *DVU2985*) for leucine biosynthesis, and three of the genes, *DVU2983* (*leuD*), *DVU2984*, and *DVU2985* (*leuB*), were significantly downregulated during salt adaptation. Two operons involved in amino acid transport were upregulated during salt adaptation, although the expression of some genes in both operons was not significantly changed. One of these operons contained three genes (*DVU2297* to *DVU2299*) and encoded a glycine/betaine/L-proline ABC transport system, and the other contained five genes (*DVU2740* to *DVU2744*) and encoded a high-affinity branched-chain amino acid ABC transporter. In addition, two other genes, *DVU0724* encoding an Na^+/Ala symporter family protein and *DVU3297* (*mtr*) encoding a tryptophan-specific transport protein, were overexpressed during salt adaptation.

We hypothesized that some amino acids, such as glutamate, tryptophan, and leucine, might play a role in *D. vulgaris* responses to a salt challenge and that import of glycine betaine into the cell from the environment could be one of the mechanisms for *D. vulgaris* osmoregulation.

(ii) Energy metabolism. There were 261 ORFs in the energy metabolism category, and significant changes were observed for 37 genes under salt adaptation conditions; the expression of 14 genes increased, and the expression of 23 genes decreased (see Fig. S2 in the supplemental material). The transcript levels for all genes (*DVU0531* to *DVU0536*) in the *hmc* operon encoding the high-molecular-weight cytochrome (HMC) increased under salt adaptation conditions (see Fig. S2 in the supplemental material). The putative transmembrane redox protein complex encoded by this operon has been reported to be involved in electron transport and energy production (24). The genes encoding a formate dehydrogenase (*DVU0587* and *DVU0588*), the periplasmic [Fe] hydrogenase (*DVU1769* and *DVU1770*), a hydrogenase expression-formation protein (*DVU1919*), and the iron-repressed flavodoxin (*DVU2680*) were also overexpressed (see Fig. S2 in the supplemental material). In contrast, the transcript levels for the genes encoding ferritin (*DVU1568*), pyruvate ferredoxin oxidoreductase (*DVU1569*, *DVU1570*, and DVU1946), pyruvate kinase (*DVU2514*), and two putative carbon starvation proteins (*DVU0598* and *DVU0599*) decreased under salt adaptation conditions (see Fig. S2 in the supplemental material). Also decreased were the transcript levels of three apparently cotranscribed genes (*glcD*, *pta*, and *ackA*) and a putative operon containing two genes (*glpF* and *glpK*) (see Fig. S2 in the supplemental material). We inferred that the electron transport in NaCl-adapted cells was much more active than that in the control cells, although carbon metabolism might have been decreased.

(iii) Sensory and regulatory genes. Significant changes in the expression of many regulatory genes during adaptation of *D. vulgaris* to NaCl suggested that a complex regulatory network was involved in cell survival during salt adaptation. Two-component signal (TCS) transduction systems are used by bacteria to sense and respond to environmental changes (53). For the *D. vulgaris* genome, 63 putative sensory histidine kinases (HK) and 66 response regulators (RR) were predicted (http://microbesonline.org/). Of the genes encoding histidine kinases and regulators, four putative sensory-regulatory genes (*rrf1*, *cckA*, *DVU2577*, and *DVU2578*) were upregulated and nine genes (*DVU0258*, *atoC*, *DVU0680*, *DVU0722*, *DVU0743*, *DVU2677*, *DVU2931*, *fexB*, and *DVU3221*) were downregulated during salt adaptation (see Fig. S3 in the supplemental material). The expression of *DVU0258* and *DVU0680* was reported to be significantly downregulated in the stationary phase in formate-based medium (64) and was also found to be decreased during salt adaptation (see Fig. S3 in the supplemental material). This similarity in regulation might reflect the slower cell growth that occurs under both conditions. The transcripts of *cheY-1* and *cheY-2* were downregulated and upregulated, respectively, under salt adaptation conditions.

The expression of 14 putative transcriptionally related genes was altered under salt adaptation conditions; 12 genes (*DVU0030*, *rrf2*, *hrcA*, *DVU1643*, *DVU1760*, *flrC*, *DVU2423*, *lysX*, *DVU2819*, *pspF*, *flrA*, and *DVU3313*) were upregulated, and only 2 genes (*phoH* and *DVU3131*) were downregulated (see Fig. S3 in the supplemental material). *rrf2* has been shown to regulate the expression of the *hmc* operon (*DVU0531* to *DVU0536*), which has been reported to be involved in energy production (24), and indeed the transcript levels of all of these genes increased under salt adaptation conditions (see Fig. S2 in the supplemental material). Similarly, *pspF* (see Fig. S3 in the supplemental material) was predicted to activate the *psp* operon, which contains 3 genes (*DVU2986* to *DVU2988*), all of which were overexpressed 5.5- to 11.4-fold under salt adaptation conditions. Psp proteins were reported to be involved in membrane integrity and the proton motive force in response to several stresses, such as heat, ethanol, pH, and osmotic shock (31, 33, 58). A recent study showed that a homolog of PspA was upregulated under high-salt conditions in the halophilic archaeon *Haloferax volcanii*, suggesting that PspA may play an important role in hypersaline adaptation in this organism (5).

(iv) Ion transport and general stress response. The predicted iron uptake and transport regulon has one four-gene operon (*feoB*, *feoA*, *DVU2573*, and *feoA*) that is predicted to be regulated by Fur (48; http://www.microbesonline.org). A previous study of *B. subtilis* also showed that there was iron deficiency and derepression of *fur* due to high salinity (28). In this study, all four genes were upregulated during both salt adaptation and salt shock (Table 2). With either NaCl treatment, the transcript levels of two genes, one predicted to be a MarR family gene (DVU0525) and the other predicted to be a drug resistance transporter gene (DVU0526), also increased. In contrast, the expression of another two-gene operon (*DVU2305* and *DVU2306*) related to phosphate transport decreased (Table 2). The results suggest that ion transporters may play important roles in the *D. vulgaris* response during salt adaptation.

Most genes involved in protein synthesis (e.g., ribosomal proteins) were repressed under salt adaptation conditions, consistent with the slower growth of salt-stressed cells. The expression of a two-gene operon (*DVU2441* and *DVU2442*) related to heat shock increased greatly, and the expression of peptidase-related genes (*DVU2568* and *DVU2569*) decreased (Table 2). The expression of *pspA* and *pspC*, which are predicted to encode phage shock proteins, was highly induced (Table 2). In addition, the expression of an operon possibly encoding bacteriophage functions predicted to contain seven genes (*DVU0198* to *DVU204*) generally decreased. For another operon consisting of three genes, the transcript levels of two of the genes (*rdl* and *perR*) increased significantly, although the *rbr* gene was not significantly upregulated ($Z = 1.09$) (Table 2). The results indicate that *D. vulgaris* also uses general strategies for survival under salt adaptation conditions.

Validation of microarray data and operon consistency by real-time RT-PCR. To verify the microarray data, 25 ORFs, including 13 ORFs in three different operons and 12 ORFs in separate operons (see Table S1 in the supplemental material) with a range of expression levels, were selected for quantitative reverse transcription (RT)-PCR (qPCR) analysis. The results showed that the microarray data were highly correlated with the qPCR measurements ($R^2 = 0.95$; $n = 38$) (see Fig. S4 in the supplemental material). This result is also consistent with the results of other studies, such as studies of nitrite stress (20) and growth transitions (14) that used the same microarray platform.

To examine if the expression of genes in the same pre-

TABLE 2. Changes in expression of genes in representative operons of *D. vulgaris* under salt adaptation conditions compared with salt shock conditions

Locus	Gene	Predicted function	Salt adaptation conditions		Salt shock conditions	
			$Log_2 R$ (treatment/control)	Z score	$Log_2 R$ (treatment/control)	Z score
Inorganic ion transport and metabolism						
DVU0525	NA[a]	Transcriptional regulator, MarR family	2.99	2.76	1.24	2.30
DVU0526	NA	Drug resistance transporter, putative	3.01	5.05	1.81	3.39
DVU2571	*feoB*	Ferrous iron transport protein B	2.87	5.09	1.88	3.54
DVU2572	*feoA*	Ferrous iron transport protein A, putative	2.11	3.21	1.98	3.60
DVU2574	*feoA*	Ferrous iron transporter component FeoA	1.21	2.13	1.46	2.31
DVU2305	NA	Conserved hypothetical protein	−2.38	−2.68	−1.07	−2.06
DVU2306	NA	Phosphate transporter family protein	−1.55	−2.89	−1.02	−1.99
General stress response						
DVU1729	NA	Killer protein, putative	2.14	2.13	2.75	4.76
DVU1730	NA	DNA-binding protein	2.46	3.36	2.28	3.91
DVU2441	*hspC*	Heat shock protein, Hsp20 family	4.01	7.42	0.94	1.67
DVU2442	NA	Heat shock protein, Hsp20 family	3.64	6.72	0.72	1.40
DVU2986	*pspC*	Phage shock protein C	2.47	4.48	0.96	1.87
DVU2987	NA	Hypothetical protein	2.90	5.42	1.15	2.22
DVU2988	*pspA*	Phage shock protein A	3.51	6.67	1.33	2.21
DVU3093	*rdl*	Rubredoxin-like protein	1.14	2.05	0.48	0.90
DVU3094	*rbr*	Rubrerythrin	0.68	1.09	0.37	0.51
DVU3095	*perR*	Peroxide-responsive regulator PerR	2.07	2.87	0.69	1.25
DVU0198	NA	Minor capsid protein C, degenerate	−2.30	−3.07	−1.36	−2.33
DVU0199	NA	Conserved hypothetical protein	−3.13	−4.22	−1.66	−2.98
DVU0200	NA	Major head protein	−1.78	−1.74	−1.27	−2.30
DVU0201	NA	Hypothetical protein	−2.85	−3.58	−1.02	−1.85
DVU0202	NA	Holin	−2.71	−3.29	−1.07	−1.96
DVU0203	NA	Conserved hypothetical protein	−2.64	−2.40	−1.20	−2.27
DVU0204	NA	Lipoprotein, putative	−2.15	−2.43	−1.38	−2.45
DVU2568	*cpsA*	Peptidase, M20/M25/M40 family	−2.03	−2.67	−0.20	−0.31
DVU2569	*slyD*	Peptidyl-prolyl *cis-trans* isomerase	−1.41	−2.05	−1.15	−2.21

[a] NA, no gene name has been assigned to the locus yet.

dicted operon is consistent, qPCR data for 13 genes (see Table S1 in the supplemental material) in three operons (http://www.microbesonline.org/) were analyzed and compared with microarray data. First, the patterns of gene expression in the three operons were consistent, but there were differences between the salt adaptation and salt shock data. For salt adaptation, no significant changes in gene expression were observed for all of the genes in the *DVU0774-DVU0780* operon, but the expression of all of the genes in both the *DVU1769-DVU1770* and *DVU2571-DVU2574* operons increased significantly (Fig. 2). For salt shock at 120 min, no significant changes in gene expression were observed for genes in the *DVU1769-DVU1770* operon, but the expression of all of the genes in both the *DVU0774-DVU0780* and *DVU2571-DVU2574* operons increased significantly (Fig. 2). Also, microarray data were positively correlated with qPCR data for both salt shock ($R = 0.94$) and salt adaptation ($R = 0.95$). In addition, the patterns of gene expression within the same operon were more similar than the patterns of expression of randomly selected genes, as determined by a computational analysis of the microarray data set as previously described (20).

Changes in amino acid levels in salt-adapted *D. vulgaris* cells. To determine if changes at the transcript level for amino acid biosynthesis genes were reflected at the metabolite level, metabolite assays were conducted with both control and salt-adapted cells. Compared to the control levels, the levels of 10 amino acids (Glu, His, Ser, Lys, Gln, Ala, Leu, Thr, and Asp) and glycine betaine increased significantly ($P < 0.01$) between 1.38- and 8-fold in NaCl-stressed cells; the level of Glu was 82.82 nmol/mg (dry weight) of cells, and the level of Ala was 17.53 nmol/mg (dry weight) of cells. The levels of six amino acids (Pro, Gly, Ile, Met, Arg, and Val) changed from 80 to 139% compared with the control levels, but no significant ($P > 0.01$) differences were observed. Notably, the levels of the three aromatic amino acids, Phe, Tyr, and Trp, appeared to decrease to 62%, 57%, and 55% of the levels in the control, respectively, but the concentrations were too low to be reliable for statistical analysis. In addition, asparagine was not detected in the control, and cysteine was not detected in the control or treated cells (Table 3). Based on both gene expression data and metabolite measurements, we suggest that the amino acids that accumulated the most (Glu and Ala) may serve as osmoprotectants during *D. vulgaris* adaptation to NaCl.

Relief of salt inhibition of *D. vulgaris* growth by external addition of amino acids. To examine the effects of amino acids on salt adaptation of *D. vulgaris*, Glu, Ala, Lys, Trp, and Leu were added individually to LS4D medium, and cell growth was monitored spectrophotometrically (OD_{600}). Significant growth effects (advantageous or deleterious) were not observed after the medium was supplemented with the amino acids compared to the control (Fig. 3A). However, when 250 mM NaCl was also added to the medium, addition of Glu, Ala, Leu, and Trp

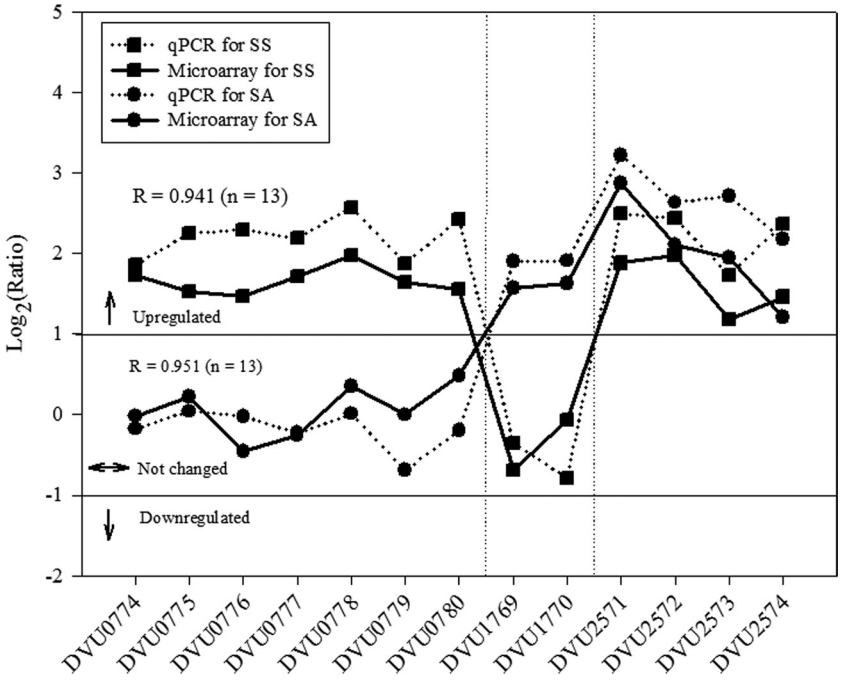

FIG. 2. Transcript levels for genes in three predicted operons detected by microarray and qPCR analyses under salt shock and salt adaptation conditions. Then two horizontal lines separate upregulated, unchanged, and downregulated genes based on a 2-fold change. The two vertical dotted lines separate three operons (*DVU0774* to *DVY0780*, *DVU1769* and *DVU1770*, and *DVU2571* to *DVU2574*) predicted by MicrobesOnline (http://www.microbesonline.org/). SS, salt shock; SA, salt adaptation.

significantly increased the growth rate, and the final OD_{600} of a *D. vulgaris* culture increased by approximately 35% after the addition of Glu. The order of growth stimulation was Glu > Ala > Leu = Trp, and no improvement in growth was observed after addition of Lys (Fig. 3B). Consistent with this observation, genes encoding some amino acid transporters (*DVU0724*, *DVU2740* to *DVU2744*, and *DVU3297*) were upregulated during salt adaptation (Table 1). We interpreted these results to mean that particular amino acids (e.g., Glu or Ala) could increase the ability of *D. vulgaris* to grow at high salinity, consistent with the accumulation of Glu and Ala under salt adaptation conditions (Table 3).

TABLE 3. Accumulation of amino acids and glycine betaine in both control and NaCl-treated *D. vulgaris* cells

Amino acid	Concn (nmol/mg [dry wt]) in:		*P* value[a]	NaCl/ control
	Control cells	NaCl-treated cells		
Glutamate	10.25 ± 0.63	82.82 ± 8.01	<0.0001	8.08
Histidine	0.07 ± 0.00	0.20 ± 0.04	<0.0001	2.74
Serine	1.30 ± 0.12	2.54 ± 0.26	<0.0001	1.95
Glycine betaine	1.40 ± 0.08	2.68 ± 0.58	0.0012	1.92
Lysine	0.15 ± 0.01	0.28 ± 0.06	0.0014	1.85
Glutamine	3.63 ± 0.38	6.48 ± 0.51	<0.0001	1.79
Alanine	9.89 ± 0.92	17.53 ± 3.89	0.0027	1.77
Leucine	0.50 ± 0.01	0.79 ± 0.05	<0.0001	1.57
Threonine	1.45 ± 0.08	2.21 ± 0.17	<0.0001	1.52
Proline	1.46 ± 0.18	2.03 ± 0.35	0.0119	1.39
Aspartate	7.41 ± 0.48	10.21 ± 1.13	0.0009	1.38
Glycine	8.86 ± 1.70	10.32 ± 3.16	0.3895	1.16
Isoleucine	0.69 ± 0.04	0.72 ± 0.02	0.1720	1.05
Methionine	0.05 ± 0.00	0.05 ± 0.01	1.0000	1.02
Arginine	0.94 ± 0.03	0.89 ± 0.03	0.0299	0.94
Valine	5.39 ± 1.43	4.30 ± 1.14	0.2193	0.80
Tyrosine	0.21 ± 0.00	0.13 ± 0.00	NT[b]	0.62
Phenylalanine	0.01 ± 0.00	0.01 ± 0.00	NT	0.57
Tryptophan	0.005 ± 0.00	0.003 ± 0.00	NT	0.55
Asparagine	ND	1.87 ± 0.41	NT	

[a] Two-tailed *P* values were obtained using Student's *t* test.
[b] NT, *t* test could not be performed since there were no standard deviations for the samples.

Comparison of effects of salt shock and salt adaptation on *D. vulgaris* gene expression. Based on a Z score cutoff (|Z|) of ≥2.0, the changes in the transcript levels of *D. vulgaris* genes in response to salt shock (at 120 min) and salt adaptation (after ca. 100 h of growth in supplemented medium) showed significant differences and similarities (Fig. 4). For the salt shock data, the 120-min time point was selected since the largest number of genes were differentially expressed at that time point (39). For the genes whose expression changed during salt adaptation, the expression of 228 upregulated ORFs (Fig. 4, region I; see Table S2 in the supplemental material) and 161 downregulated ORFs (Fig. 4, region II; see Table S3 in the supplemental material) did not change significantly under salt shock conditions. Similarly, for the genes whose expression changed during salt shock, the expression of 154 upregulated ORFs (Fig. 4, region III; see Table S4 in the supplemental material) and 99 downregulated ORFs (Fig. 4, region IV; see Table S5 in the supplemental material) did not change during salt adaptation. On the other hand, a comparison of the transcriptome profiles for salt adaptation and salt shock indicated that there were some similarities; under both conditions the expression of 72 ORFs increased (Fig. 4, region V; see Table S6 in the supplemental material) and the expression of 53

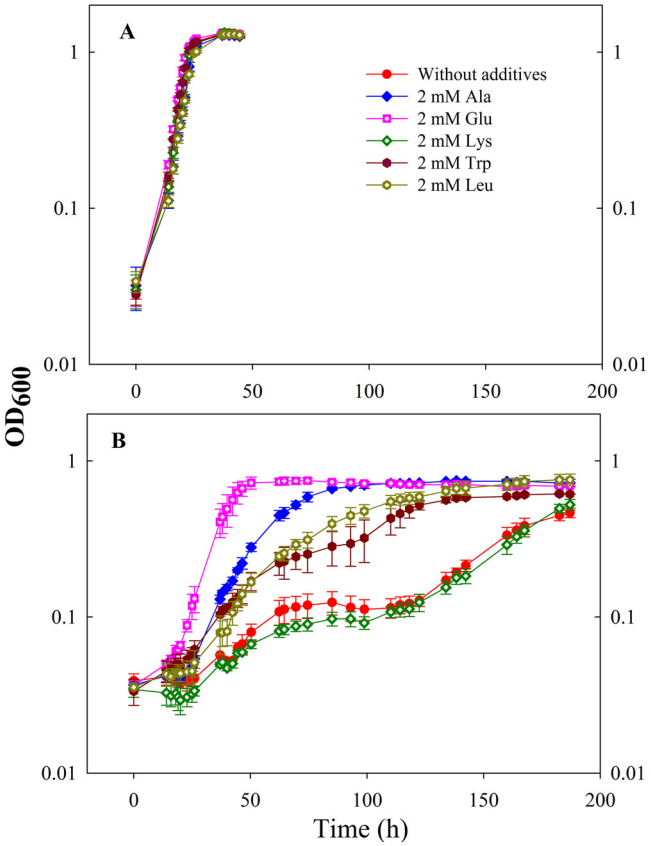

FIG. 3. Relief of salt inhibition by external addition of the amino acids Glu, Ala, Leu, Trp, and Lys to LS4D medium under the control conditions (A) and under stress conditions (250 mM NaCl) (B). *D. vulgaris* cells were grown at 37°C, and their growth was monitored by determining the OD_{600}.

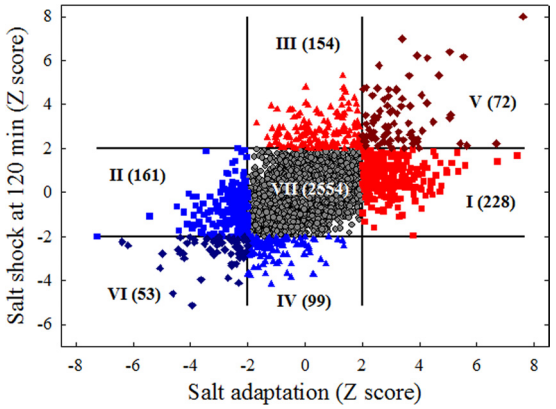

FIG. 4. General comparison of global transcriptome profiles of *D. vulgaris* under salt adaptation and salt shock conditions. The Z score cutoff for significant changes (|Z|) was set to ≥2.0. A total of 3,321 ORFs were detected for both stress conditions, and all of the Z scores are shown. The *x* axis shows data for salt adaptation, and the *y* axis shows data for salt shock. Region I, upregulated genes under salt adaptation conditions; region II, downregulated genes under salt adaptation conditions; region III, upregulated genes under salt shock conditions; region IV, downregulated genes under salt shock conditions; region V, upregulated genes under both conditions; region VI, downregulated genes under both conditions; region VII, genes that changed insignificantly. The numbers in parentheses are the numbers of genes in the categories.

ORFs decreased (Fig. 4, region VI; see Table S7 in the supplemental material). The genes that were differentially expressed under both conditions accounted for only 16.3% of the 767 genes with significant changes for the two conditions combined.

A comparison of transcriptome profiles for amino acid biosynthesis by *D. vulgaris* under salt adaptation and salt shock conditions showed that a cell might develop complex response mechanisms. First, the genes predicted to be involved in the synthesis of Glu, Trp, and Leu were different for the two treatments. *DVU0375*, potentially encoding glutamate dehydrogenase, was upregulated under salt adaptation conditions, while *gltB-1* and *DVU3291*, encoding a putative glutamate synthase, were upregulated under salt shock conditions; *trpB-1*, *trpB-2*, and *trpA* were all upregulated under salt shock conditions, but only *trpB-1* was upregulated under salt adaptation conditions; and five genes in the predicted operon for Leu synthesis exhibited a trend toward upregulation under salt shock conditions but a trend toward downregulation under salt adaptation conditions (Table 1). Similarly, several amino acid transport genes responded in opposite ways under salt adaptation and salt shock conditions. For example, the expression of *DVU0724*, *DVU2341*, and *mtr* increased during salt adaptation but did not change under salt shock conditions (Table 1). The results suggest that *D. vulgaris* uses different strategies to accumulate osmoprotectants to cope with osmotic stress under salt adaptation and salt shock conditions.

Second, efflux systems, including Na^+-H^+ antiporters, and cation/multidrug resistance proteins may pump cations (e.g., Na^+) out of the cell, and the genes encoding these proteins in three operons (*DVU0060* to *DVU0063*, *DVU2815* to *DVU2817*, and *DVU3326* and *DVU3327*) exhibited a trend toward upregulation under both salt adaptation and salt shock conditions (see Fig. S5 in the supplemental material). *DVU0060* encodes an efflux transporter; *DVU0061*, *DVU2816*, *DVU2817*, *DVU3326*, and *DVU3327* encode multidrug resistance proteins; and *DVU0062* and *DVU2815* encode outer membrane proteins. However, a putative three-gene operon (*DVU0057* to *DVU0059*), with *DVU0058* encoding an efflux transporter and *DVU0059* encoding an AcrB/AcrD/AcrF family protein, was significantly upregulated only under salt shock conditions (see Fig. S5 in the supplemental material). In addition, the differential expression of these genes was generally higher under salt shock conditions than under salt adaptation conditions (see Fig. S5 in the supplemental material). Consistent with the high energy requirement for efflux processes, the F-type ATPase genes (*DVU0774* to *DVU0780*) were observed to be upregulated under salt shock conditions but not under salt adaptation conditions (see Fig. S5 in the supplemental material). The results suggest that exclusion of Na^+ from the cell might be a more important mechanism for an initial response to salt shock than for salt adaptation in *D. vulgaris*.

Third, most genes involved in carbon metabolism were downregulated under both salt shock and salt adaptation conditions. For example, all of the genes in the operon containing *DVU3025*, *DVU3026*, *DVU3027*, *DVU3028*, *DVU3029*, and *DVU3030*, which encode a putative pyruvate-ferredoxin oxi-

doreductase, an L-lactate permease family protein, the GlcD subunit of glycolate oxidase, an iron-sulfur cluster-binding protein, a phosphate acetyltransferase, and an acetate kinase, respectively, were generally downregulated under both salt shock and salt adaptation conditions (see Fig. S2 in the supplemental material). Additionally, more genes involved in carbon metabolism, including *porA* and *porB* encoding the alpha and beta subunits of pyruvate ferredoxin oxidoreductase, *corB* encoding the putative beta subunit of pyruvate ferredoxin oxidoreductase, *glpF* encoding a glycerol uptake facilitator protein, *glpK* encoding a glycerol kinase, and *DVU3349* encoding a putative pyruvate flavodoxin-ferredoxin oxidoreductase, were downregulated under salt adaptation conditions, but their expression did not change significantly under salt shock conditions (see Fig. S2 in the supplemental material). The results suggest that the decreased carbon metabolism was consistent with slower growth during salt adaptation and possibly redirection of carbon flow to the generation of energy for exclusion of Na^+ and the production of osmoprotectants.

Fourth, two chemotaxis genes (*DVU0048* and *cheV-3*) were downregulated under salt adaptation conditions, and one gene (*DVU1458*) exhibited lower expression during salt shock; similarly, four methyl-accepting chemotaxis genes (*DVU0170*, *mcpD*, *DVU0608*, and *DVU2585*) were downregulated under salt adaptation conditions, and one gene (*DVU0608*) was downregulated during salt shock (see Fig. S5 in the supplemental material). In addition, only one gene (*DVU1884*) was found to be upregulated under salt adaptation conditions, and no gene was found to be upregulated under salt shock conditions (see Fig. S5 in the supplemental material). The results suggest that escape from stressful conditions was not a major strategy under the salt adaptation conditions, although it could be an initial response to salt shock. However, the expression of most genes involved in flagellar biosynthesis was not significantly changed under salt shock or salt adaptation conditions; only one gene (*DVU3230*) during salt adaptation and another gene (*DVU3231*) during salt shock were downregulated (see Fig. S5 in the supplemental material), suggesting that the general motility of a cell may not be significantly changed under either salt adaptation or salt shock conditions.

Fifth, nearly all significant changes in the expression of regulatory genes of *D. vulgaris*, such as *rrf2*, *DVU1645*, *pspF*, *flxB*, and *cheY-2*, under salt shock conditions could also be observed under salt adaptation conditions (see Fig. S3 in the supplemental material). However, the expression of more genes was changed only under salt adaptation conditions; some examples are *cckA*, *flrC*, *lysX*, *DVU3313*, and *cheY-1* (see Fig. S3 in the supplemental material). These results indicate that *D. vulgaris* adaptation to elevated NaCl levels requires coordination of more genes, perhaps via a more complicated regulatory network.

Finally, *D. vulgaris* may also use general stress response strategies to deal with salt adaptation and salt shock, which was reflected in the similar expression profiles for genes involved in amino acid synthesis and transport, such as *trpB-1*, the three-gene operon encoding the glycine betaine/L-proline ABC transporter, and the five-gene operon encoding the high-affinity branched-chain amino acid ABC transporter (Table 1). Other similarities are shown in Fig. S2 in the supplemental material. The results suggest that these processes are critical for the response of *D. vulgaris* to salt shock and for salt adaptation.

DISCUSSION

In this study, we examined the adaptation of *D. vulgaris* to high salinity by performing transcriptome, growth, and metabolite analyses. The global transcriptome profile identified groups of genes involved in amino acid biosynthesis and transport, energy metabolism, regulatory functions, and general stress responses that may be important for salt adaptation. Metabolite measurement revealed that amino acids, such as glutamate, might be used as osmoprotectants in *D. vulgaris*, and this notion was supported when external addition of glutamate relieved salt inhibition. A comparison of transcriptome profiles suggested that *D. vulgaris* may use different strategies to deal with the initial salt shock and subsequent salt adaptation. A conceptual model was constructed to provide an integrated understanding of the mechanisms of *D. vulgaris* adaptation to elevated NaCl levels.

One of the major strategies for bacterial survival and growth under osmotic and/or salt stress conditions is accumulation of compatible solutes, such as amino acids, by import or synthesis. Our previous study (39) suggested that *D. vulgaris* imported osmoprotectants (e.g., glycine betaine) mainly to deal with salt shock, which may also be an initial mechanism for salt adaptation. A few amino acid transport genes (e.g., *proW*, *livF*, *livM*, and *DVU2744*) were highly upregulated. High-affinity transporters import amino acids even when their concentrations in the medium are low. Accumulation of glutamate was found to be an important osmoregulatory mechanism in response to osmotic and/or salt stresses in bacteria, and this study shows that this may also be true in *D. vulgaris*. Glutamate is a central factor in global nitrogen metabolism (47, 62). It appears that genes encoding both the GS-GOGAT cycle through glutamine synthetase and glutamate synthase and the GDH pathway through glutamate dehydrogenase for glutamate biosynthesis are present in the *D. vulgaris* genome (http://www.microbesonline.org/). No specific glutamate dehydrogenase (GDH) gene has been assigned in *D. vulgaris*. However, two genes (*DVU0375* and *DVU0964*) are annotated as genes encoding Glu/Leu/Phe/Val dehydrogenase family proteins, and indeed, *DVU0375* was significantly upregulated under salt adaptation conditions. One gene (*DVU1258*) encoding a putative glutamine synthetase (GS) and five genes (*DVU1821* to *DVU1823*, *DVU2476*, and *DVU3291*) encoding various subunits of glutamate synthase (GOGAT) are present in the genome, but their expression did not significantly change under salt adaptation conditions. In contrast, there was a trend toward overexpression of *DVU1821*, *DVU1823* (there were no data for *DVU1822*), and *DVU3291* during salt shock (39). It is known that the GS-GOGAT pathway consumes 1 ATP for the production of 1 mol of glutamate from 1 mol each of NH_3, 2-oxoglutarate, and NADPH and is energetically more costly than the GDH pathway. In *E. coli*, the GDH pathway is used when the energy in the cell is limited but excess ammonium and excess phosphate are present (25). Therefore, these observations suggest that *D. vulgaris* uses mainly the GS-GOGAT pathway under salt shock conditions and the GDH pathway to save energy under salt adaptation conditions for glutamate

synthesis. However, further studies are required to confirm this hypothesis.

Although the intracellular concentration of Ala reached 17.53 nmol/mg (dry weight) of cells and one gene (*DVU0724*) predicted to encode a sodium/alanine symporter family protein was also upregulated, there have been only a few reports indicating that Ala or the other accumulated amino acids (e.g., Leu, Ser, Gln, and Lys) serve as osmoprotectants in bacteria. One example is the finding that the nitrogen-fixing cyanobacterium *Anabaena* sp. 287 exhibited a significant increase in tolerance to NaCl in the presence of Ala or other amino acids, such as Val, Pro, Lys, and His, and this study also showed that Ala relieved the initial inhibition by salt of enzymes involved in nitrogen fixation, photosynthesis, and respiration (56). In growth assays, addition of 2 mM Ala partially alleviated the inhibitory effect of NaCl, which confirmed that accumulation of Ala could provide protection against an osmotic challenge.

Of all the amino acids tested, only Lys did not appear to have a mitigating effect on growth. However, the data presented here do not provide insight into the mechanisms by which the accumulation or addition of amino acids can alleviate salt inhibition, and further investigations are required to understand these mechanisms. The results also indicate that *D. vulgaris* does accumulate compatible solutes, such as glutamate, as an important strategy for survival when it is grown with high levels of salt.

The transcriptome data suggest that there is a decrease in carbon metabolism under salt adaptation conditions, and this was reflected mainly in the lower growth rate and in the downregulation of carbon utilization genes and putative carbon starvation genes. First, although the amounts of the membrane-bound lactate dehydrogenase (DVU0600; Z score, −1.2) and the primary pyruvate:ferredoxin oxidoreductase (DVU3025; Z score, −1.5) were not significantly altered, the expression of genes apparently encoding a second pyruvate ferredoxin oxidoreductase (PorA, PorB, OorB, and DVU3349) was significantly lower under salt adaptation conditions. Second, the expression of the *pta* and *ackA* genes was also significantly downregulated, which might have resulted in decreased energy production via substrate-level ATP synthesis. In addition, two genes encoding putative carbon starvation proteins were also significantly downregulated, suggesting that *D. vulgaris* may utilize less of a carbon source under salt adaptation conditions, which is consistent with the slow cell growth.

However, electron transport processes appeared to be more active or unchanged, which is supported by the expression of genes involved in both hydrogen cycling (42) and formate cycling (24). In the proposed hydrogen cycle model, periplasmic and cytoplasmic hydrogenases are required to form a proton gradient for ATP synthesis and transport processes. In this study, the expression of a periplasmic hydrogenase (DVU1769 and DVU1770) was upregulated during salt adaptation, although the transcript levels of the genes encoding the two putative cytoplasmic hydrogenases (Ech and Coo) were not significantly increased. Similarly, formate cycling could also generate a proton gradient and provide energy for transport processes and ATP synthesis (24). Indeed, two genes (*DVU0587* and *DVU0588*) encoding a formate dehydrogenase (Fdh) were upregulated under salt adaptation conditions in this study. Interestingly, the expression of genes for sulfate reduction, which occurs in the cytoplasm, remained unchanged even though the expression of genes for a hydrogenase, which occurs in the periplasm, seemed to be increased. One possibility is that such electron transport processes are modulated by the complicated *c*-type cytochrome network in *D. vulgaris* (24) and so the genes for sulfate reduction were not responsive.

In the heterotrophic bacteria *E. coli* and *B. subtilis* the expression of salt-inducible genes is regulated by the sigma factors RpoS and SigB, respectively (7, 22, 23, 27). In the photoautotrophic bacterium *Synechocystis* sp. PCC 6803, a few HKs have been identified as sensors for salt stress (37). However, so far, *D. vulgaris* appears not to have an RpoS ortholog, similar sensors, or regulators reported to be responsible for salt adaptation. Fur is considered to be a global regulator in *D. vulgaris* (26). It was observed that high salinity caused iron deficiency and led to derepression of Fur-regulated genes in *B. subtilis* (28). Although the expression of *fur* did not change significantly in *D. vulgaris* under salt adaptation conditions, the expression of some genes predicted to be regulated by Fur, such as *DVU0273*, *DVU2574* (*feoA*), *DVU2680*, and *DVU3330* (48), did increase under salt adaptation conditions. Similar results were also observed for *D. vulgaris* under nitrite stress (20) and salt shock (39) conditions. Thus, Fur, a potential global regulator, may also play an important role in *D. vulgaris* under salt adaptation conditions. The homolog of *fur*, *perR* (*DVU3095*), was also significantly upregulated under salt adaptation conditions. PerR is predicted to regulate oxidative stress genes and also responds to iron concentrations (11, 12). *rdl* (*DVU3093*), which encodes a rubredoxin-like protein and is predicted to be regulated by PerR, was overexpressed during salt adaptation. However, since the functions of most regulatory genes are unknown, identification of regulatory networks in *D. vulgaris*, as well as in other organisms, in response to salt adaptation remains a great challenge.

Generally, the responses of *D. vulgaris* to elevated NaCl levels may follow a temporal pattern from salt shock to salt adaptation. This was first reflected in the changes in the expression of specific genes involved in the import of osmoprotectants, the efflux of harmful ions, the energy supply, and cell motility. At an initial stage, *D. vulgaris* may import effective osmoprotectants, exclude Na^+ from the cell, and move away from the stressful environment. The dynamic change in the expression of chemotaxis genes is a good example. At early time points during salt shock, many chemotaxis-related genes were found to be upregulated; at later time points during salt shock, only a few such genes were overexpressed, and the expression of most genes remained unchanged (39). When *D. vulgaris* was exposed to persistent NaCl stress, only two chemotaxis genes were upregulated, and most of the chemotaxis genes were downregulated. A dynamic process was also seen for the accumulation of Glu. For example, the concentration of Glu increased more than 8-fold during salt adaptation but increased only about 2-fold during salt shock (39). In addition, the expression of regulatory genes also showed a similar trend, and the expression of more genes was observed to be changed during salt adaptation than during salt shock.

Considering the data and interpretations described above, we propose a simple conceptual model for *D. vulgaris* adaptation to elevated NaCl levels (Fig. 5). Salt stress may be sensed by unknown HKs and then transduced to regulators, which

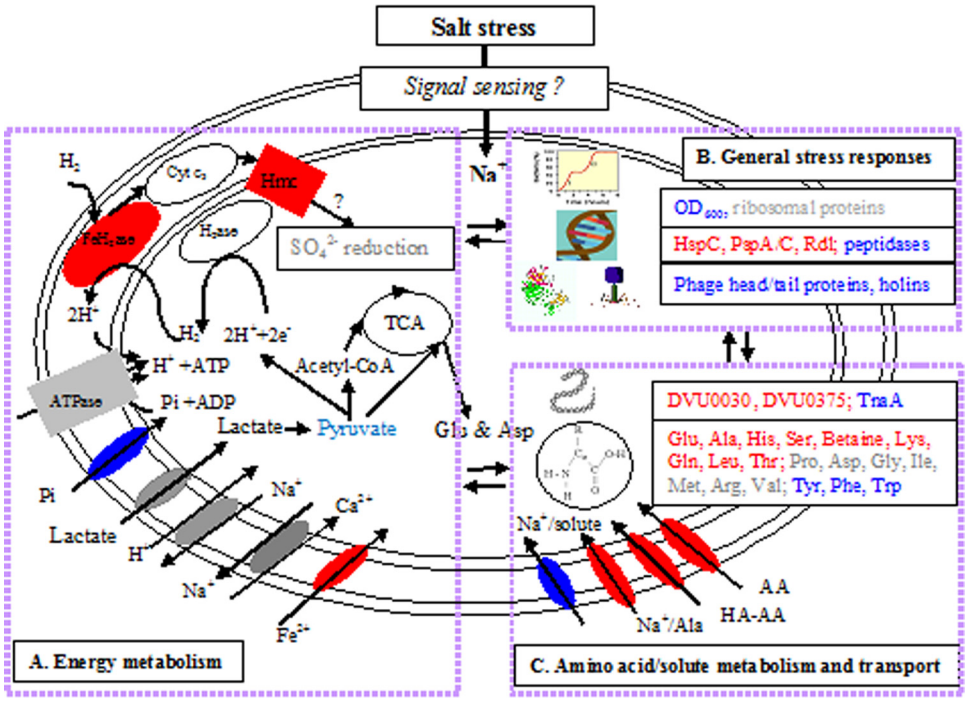

FIG. 5. Conceptual model of *D. vulgaris* Hildenborough responses to a long-term exposure to NaCl. Red, upregulation or increase; blue, downregulation or decrease; gray, no significant change. (A) Energy metabolism. (B) General stress response. (C) Protein, amino acid, and solute metabolism and transport. Cyt c_3, cytochrome c_3; TCA, tricarboxylic acid cycle; CoA, coenzyme A; AA, amino acid; HA-AA, high-affinity amino acid.

regulate cellular activities of *D. vulgaris* in response to high salinity. In this model, only three categories of cellular activities (energy metabolism, general stress response, and amino acid or solute metabolism and transport) are emphasized based on our experimental results from growth, physiological, transcriptome, and metabolite analyses. First, the transport of iron, phosphate (P_i), and amino acids into the cell and the exclusion of Na^+ from the cell require ATP hydrolysis. Although the transcriptome data suggest that there is a reduction in ATP generation from the oxidation of lactate and pyruvate during salt adaptation, the overexpression of genes related to hydrogen oxidation and electron transport suggests that the levels of electron flow components may be increased. Interestingly, sulfate reduction was unchanged under salt stress conditions (Fig. 5A).

Second, the most significant responses of *D. vulgaris* to salt adaptation were decreased cell growth and overexpression of genes related to heat shock, phage shock, and oxidation. The latter response is important for *D. vulgaris* to maintain the integrity of protein and membrane structures, prevent oxidative stress, suppress bacteriophage development (e.g., downregulation of bacteriophage related genes), and then adapt to salt stress conditions (Fig. 5B).

Third, the accumulation of small molecules (e.g., amino acids) to counter osmotic stress is one of the most important mechanisms in *D. vulgaris*. Our data strongly suggest that biosynthesis or import of one or more particular amino acids is the primary mechanism for salt adaptation. One of these amino acids, glutamate, may be the most effective amino acid for adaptation of *D. vulgaris* to a high-salinity environment (Fig. 5C).

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