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Global Protein-Level Responses of *Halobacterium salinarum* NRC-1 to Prolonged Changes in External Sodium Chloride Concentrations

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Responses to changes in external salinity were examined in *Halobacterium salinarum* NRC-1. *H. salinarum* NRC-1 grows optimally at 4.3 M NaCl and is capable of growth between 2.6 and 5.1 M NaCl. Physiological changes following incubation at 2.6 M NaCl were investigated with respect to growth behavior and proteomic changes. Initial observations indicated delayed growth at low NaCl concentrations (2.6 M NaCl), and supplementation with different sugars, amino acids, or KCl to increase external osmotic pressure did not reverse these growth perturbations. To gain a more detailed insight into the adaptive responses of *H. salinarum* NRC-1 to changes in salinity, the proteome was characterized using iTRAQ (amine specific isobaric tagging reagents). Three hundred and nine differentially expressed proteins were shown to be associated with changes in the external sodium chloride concentration, with proteins associated with metabolism revealing the greatest response.

Keywords: iTRAQ • changed osmotic conditions • halophilic Archaea • proteomics • mass spectrometry

Introduction

Biological systems have evolved mechanisms to appropriately respond to environmental stresses that can damage proteins and DNA.[1] A very common stress situation is the change in external osmolarity due to extended periods of drought or rain. Members of the family *Halobacteriaceae* are particularly vulnerable to decreases in external salinity, as they need at least 1.0−1.5 M NaCl (∼6 to 9% w/v) for growth.[2] To avoid lysis under low-osmolarity or dehydration under high-osmolarity growth conditions, halophilic archaea possess active mechanisms that permit timely and effective adaptation to changes in the molecular concentrations of the environment.[3]

Although halophilic archaea typically thrive in hypersaline environments, recent studies have described halophilic archaea from low-osmotic environments, for example, Zodletone spring with 0.7−1% (w/v) NaCl,[4] the colne salt marshes with 2.5% (w/v) NaCl,[5] and modern stromatolites (∼6% (w/v) NaCl).[6,7] These findings indicate a much broader environment for halophilic archaea than previously thought. It is therefore of great interest to investigate what cellular mechanisms are utilized by halophilic archaea to withstand changes in external osmotic and/or salinity conditions.

One of the best-studied members of the family *Halobacteriaceae* is *Halobacterium salinarum* NRC-1 (formerly *Halobacterium* sp. NRC-1, recently amended[8]) with the genome sequence published in 2000.[9] *H. salinarum* NRC-1 belongs to the genus *Halobacterium*, the type genus of the family *Halobacteriaceae*.[10] To balance external osmotic pressure, halophilic archaea typically generate high intracellular concentrations of inorganic cations (predominantly K^+). Recent studies have revealed that some halophilic archaea (*H. salinarum*), however, can also, or alternatively, accumulate compatible solutes, such as trimethylammonium compounds, to balance their internal osmotic pressure.[11] Previously, this and other studies have focused on the response of *H. salinarum* NRC-1 exposed to different environmental stress situations, including UV radiation,[1,12,13] heat shock,[14] and changes in sodium chloride concentrations.[15−18]

Here, we have examined the overall proteomic response to changes in external sodium chloride concentrations in *H. salinarum* NRC-1 employing isobaric tagging for relative and absolute protein quantification (iTRAQ). iTRAQ predominately labels primary amines and ε-amines of lysine, which allows the quantification of suitable peptides present within the sample.[19,20] The reagents are differentially isotopically labeled such that all derivatized peptides are isobaric and chromatographically indistinguishable, yet yield signature or reporter ions following CID (Collision Induced Dissociation) that can be used to identify and quantify individual members of the multiplex set.[20] Furthermore, the possibility of multiplexing the analysis with up to eight samples in a single experiment allows the direct comparison between different physiological stages.

The focus of this study was the response of *H. salinarum* NRC-1 to altered salinity, as a component of the adaptation to changed osmotic conditions. Aspects of the general response, such as growth patterns, cell recovery, and protein synthesis,

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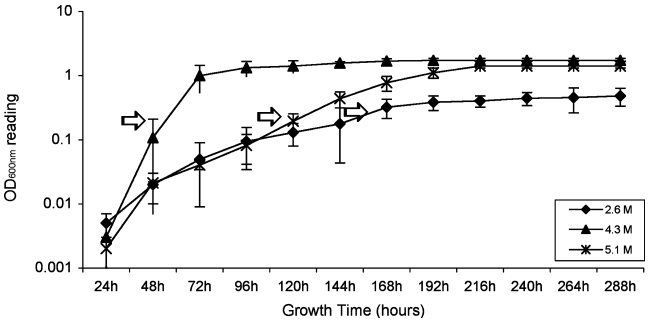

Figure 1. Growth profile of *H. salinarum* NRC-1 incubated at 2.6, 4.3, and 5.1 M NaCl, respectively. Arrows indicate time points of sampling.

with the particular analysis of proteomic changes, have been investigated and their significance discussed.

Experimental Procedures

Culture Conditions and Growth Studies. *H. salinarum* NRC-1 was a kind gift from Prof. Stan-Lotter. *H. salinarum* NRC-1 was cultivated under aerobic conditions with exposure to room light in 20 mL of ATCC 2185 media containing 4.3 M NaCl (optimum NaCl concentration) at 37 °C on a rocking platform (160 rpm) for 5 days. Subsequently, 100 μL of this culture was taken and transferred (in triplicate) into fresh ATCC 2185 media containing 2.6 M NaCl (low osmotic condition), 4.3 M NaCl (optimal growth condition), and 5.1 M NaCl (high osmotic condition), respectively. These starter cultures were incubated under identical conditions for up to 1 week. To adapt *H. salinarum* NRC-1 to these changed conditions, 100 μL of 2.6, 4.3, and 5.1 M NaCl cultures was inoculated in 20 mL of fresh media containing 2.6, 4.3, and 5.1 M NaCl, respectively. This subculturing was repeated twice to obtain adapted cultures. Triplicate cultures were then grown to an OD_{600nm} of 0.4−0.5. Samples were washed three times with a TN buffer (100 mM Tris; 10 mM EDTA, pH 7.4; and either 2.6, 4.3, or 5.1 M NaCl, depending on the salt concentration of the cultivation media).

Furthermore, cultures incubated at 2.6 M NaCl were supplemented with betaine, glycine, alanine, histidine (0.1% w/v), maltose, fumerate, sucrose, glucose, lactose, sorbitol, trehalose, arabinose, xylose, raffinose, and cellobiose (1% w/v) to evaluate if supplementing with these substances can restore normal growth patterns.

Protein Isolation, Purification, and iTRAQ Labeling. Triplicate cultures for each osmotic condition were grown in 2.6, 4.3, or 5.1 M NaCl, respectively (Figure 1) and proteins were recovered from each culture as previously described.[21] Soluble proteins were recovered by centrifugation (10 min at 10 000*g*) of cell lysate, which was prepared by resuspending the cell pellet in 1 mL of water and 1 mM PMSF. The insoluble proteins were dissolved in 3 mL of 10% SDS and centrifuged again (10 min at 10 000*g*). Both soluble and insoluble protein fractions were combined and treated with 20 U DNase (37 °C for 30 min) to remove nucleic acids and acetone-precipitated. iTRAQ labeling was conducted as per manufacturer's instructions (Applied Biosystems) with some modification. $NaHCO_3$ (1 M) was used as dissolution buffer and cysteines were alkylated with 10 mM iodoacetamide. Samples extracted from 2.6 M NaCl were labeled with iTRAQ reagent 115, samples extracted from 4.3 M NaCl were labeled with iTRAQ reagent 116, and samples extracted from 5.1 M NaCl were labeled with iTRAQ reagent 117 (Figure 2). The pH was maintained at around 8.1 by adding 1−2 μL of $NaHCO_3$ (~25 mM) throughout the reaction. Samples were diluted with 3 mL of load buffer and 25 μL of glacial acetic acid to reduce the salt content and pH sufficiently to allow successful strong cation exchange (SCX) chromatography.[19]

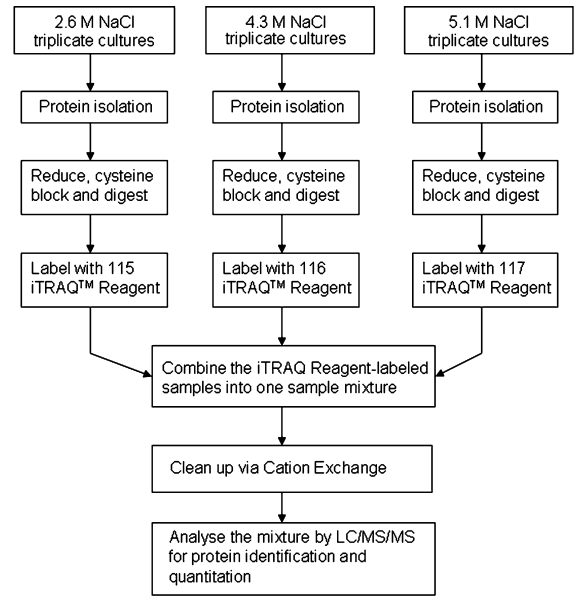

Figure 2. A schematic overview of iTRAQ labeling.

Two-Dimensional Liquid Chromatography and Mass Spectrometry. Samples were first desalted using Stage Tips (Proxeon, Odensa, Denmark) and lyophilized. iTRAQ samples were separated by automated online strong cation exchange (SCX) and nano C18 LC using an Ultimate HPLC, Switchos and Famos autosampler system (LC-Packings). Peptides (~1−2 μg in 0.05% HFBA) were loaded onto a SCX micro column (0.75 × ~20 mm, Poros S10, Applied Biosystems) and the eluant from multiple salt elution steps (unbound load, 5, 10, 15, 20, 25, 30, 40, 50, 100, 250, 500, and 1000 mM ammonium acetate) was captured and desalted on a C18 precolumn cartridge (500 μm × 2 mm, Michrom Bioresources). After a 10 min wash, the precolumn was switched (Switchos) into line with a fritless analytical column (75 μm × ~12 cm) containing C18 RP packing material (Magic, 5 μm, 200 Å).[22] Peptides were eluted using a linear gradient of buffer A (98:2, H_2O/CH_3CN, 0.1% formic acid) to 45% buffer B (20:80, H_2O/CH_3CN, 0.1% formic acid) at ~300 nL/min over 75 min. High voltage (2300 V) was applied through a low volume tee (Upchurch Scientific) and the tip was positioned ~1 cm from the orifice of an API QStar Pulsar i hybrid tandem mass spectrometer (Applied Biosystems, Foster City, CA). Positive ions were generated by electrospray and the QStar operated in information-dependent acquisition mode (IDA). A Tof MS survey scan was acquired (m/z 350−1700, 1 s) and the 3 largest multiply charged ions (counts >20, charge state ≥2 and ≤4) were sequentially selected by Q1 for MS-MS analysis. Nitrogen was used as collision gas and an optimum collision energy was automatically chosen (based on charge state and mass). Tandem mass spectra were accumulated for up to 2.5 s (m/z 65−2000) with 2 repeats. Peak lists were generated using Mascot Distiller (Matrix Science, London, England) using the default parameters, and submitted to the database search program Mascot (version 2.2, Matrix Science). Search parameters were Precursor and product ion tolerances

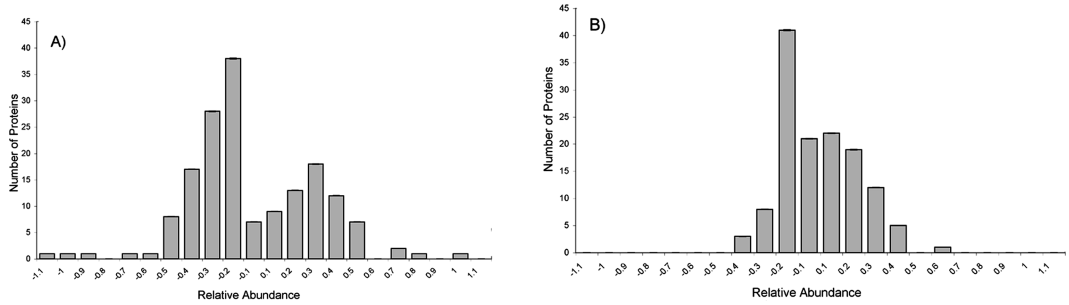

Figure 3. Differential expression of all labeled proteins in log(10) scale. Relative abundance higher than 0 indicates up-regulation of proteins; relative abundance lower than 0 indicates down-regulation.

±0.25 and 0.2 Da, respectively; Met(O), Cys-carboxyamidomethylation and iTRAQ-4-plex reagents on the N-terminus, Lys and Tyr were specified as variable modification, enzyme specificity was trypsin, 1 missed cleavage was possible, and the $NCBI_{nr}$ database (Oct 2007) was searched. High scores indicated a likely match.

Data Analysis. Retrieved data was analyzed using programs ProQuant (V1.0) and Pro Group Viewer (V1.05) (Applied Biosystems). For the ProQuant analysis, the cutoff confidence setting was 99%. A mass deviation of 0.15 Da for precursor and 0.15 Da for fragment ions was permitted during the analysis against the *H. salinarum* NRC-1 database. For each protein, the Pro Group Algorithm reports an unused ProtScore and a total ProtScore.[23] The total ProtScore is a measurement of all the peptides evidence for a protein, whereas the unused ProtScore is a measurement of all the peptides evidence for a protein that is not better explained by a higher ranking protein.[23] In short, the unused ProtScore is calculated using unique proteins (peptides that have not been linked to higher ranking proteins), and is a true indicator of protein evidence.[23] For this study, the unused ProtScore setting was 2.0. Significant changes in regulation are given as the log(10) of the average ratio of the relative quantification of peptide ions. Relative abundance higher than 0 indicates up-regulation; lower abundance than 0 indicates down-regulation.

To be able to estimate the false-positive rate of peptide identification by Mascot, a random database containing shuffled proteins from *H. salinarum* NRC-1 was created.[19,24] Estimating the false-positive rate is possible using the expression %false $= 2[\eta_{shuffle}/(\eta_{shuffle} + \eta_{observed})]$.[19] To determine the differential expression of proteins between 2.6, 4.3, and 5.1 M NaCl, respectively, the mean ratio of identified proteins was calculated. The *p*-value was set to $p < 0.05$ (5%) of level of significance as a criterion for the analysis of differentially expressed proteins. Standard deviation and 99% confidence interval were also calculated for each protein ratio within the significant range of entries.

Functional and Structural Prediction of Hypothetical Proteins. Protein-fold predictions were used to assign functions to previously unannotated proteins. Consensus fold recognition searches a query sequence for a match to a known structure using a number of methods, including threading and fold-recognition. The results from several methods are then collected and compiled into a single consensus structure.[1] For this investigation, the Web-based fold recognition server PCons/PModel was used.[25,26]

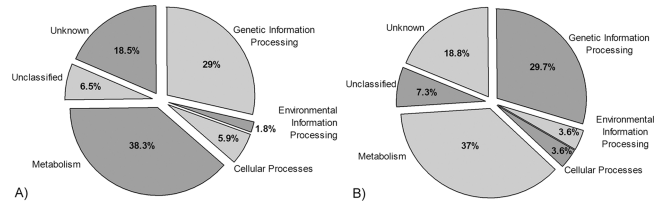

Figure 4. Distribution of all proteins identified after iTRAQ labeling into different functional categories according to the KEGG database.

Results and Discussion

Preliminary Protein Expression in Stressed versus Adapted Cultures. *H. salinarum* NRC-1 was exposed to the upper and lower limits of NaCl concentrations suitable for growth. Supplementing KCl (10 g l^{-1} compared to 3 g l^{-1} in the original media), in order to counterbalance the reduction in external NaCl, had no positive effect on growth under low-osmotic conditions (2.6 M NaCl). No effects were also observed upon supplementation with betaine, glycine, alanine, histidine (0.1% w/v), maltose, fumerate, sucrose, glucose, lactose, sorbitol, trehalose, arabinose, xylose, raffinose, or cellobiose (1% w/v). Re-cultivation in an attempt to adapt cultures to the different osmotic conditions did not improve growth patterns. Protein abundance changes were measured following recultivation of *H. salinarum* NRC-1 in 2.6 and 5.1 M NaCl, and compared to optimum growth at 4.3 M NaCl.

System Level Analysis of Proteome Changes in Response to Sodium Chloride Levels. Three replicate iTRAQ experiments were conducted and the data combined into one set using the software ProQuant and ProGroup Viewer. In total, 588 proteins with a confidence of >95% were identified. The *Halobacterium* sp. NRC-1 genome encodes 2630 predicted proteins;[9] thus, the iTRAQ data set represented 22.35% coverage of the theoretical proteome. Of these 588 proteins, 309 showed differential expression following either low (2.6 M NaCl) or high (5.1 M NaCl) osmotic conditions (Figure 3). The complete list of all proteins identified is provided in the Supporting Information. All identified proteins were validated by searches using the Mascot database and the false-positive rate was determined as previously described.[19] In total, 2152 high scoring peptides (Mowse score: 30) were identified by searching the true database, and 17 high scoring peptides (Mowse score: 30) were identified by searching the shuffled database. The false-positive rate derived from the Mascot searches with different Mowse scores was calculated to be <0.5%. Therefore, proteins identified with a Mowse score <30 were excluded from the analysis. Identified proteins were separated into different functional groups according to the KEGG database (Figure 4, Tables 1 and 2, and Supporting Information Tables 1 and 2).[27]

Table 1. Proteins That Showed Significant Alteration Following Low (2.6 M NaCl) Osmotic Conditions

KEGG classification	gene locus	protein	annotation	log10 ratio[a]
Cellular Processes and Signaling	VNG0960G	FlaB1	Flagellin B1 precursor	−0.3465 ± 0.05
	VNG0961G	FlaB2	Flagellin B2 precursor	−1.0391 ± 0.65
	VNG0962G	FlaB3	Flagellin B3 precursor	−0.5235 ± 0.55
	VNG1009G	FlaA2	Flagellin A2 precursor	−0.6953 ± 0.10
	VNG1339H	VNG1339H	Hypothetical protein	0.3410 ± 0.19
	VNG1467G	Bop	Bacteriorhodopsin	0.3185 ± 0.21
	VNG1659G	Htr1	Hrt1 transducer; Methyl-accepting chemotaxis protein	−0.3984 ± 0.05
	VNG2378G	NosF1	Copper transport ATP-binding protein	0.3655 ± 0.23
Environmental Information Processing	VNG2086G	Hpb	ABC-type phosphate transporter	−0.246 ± 0.09
	VNG2349G	DppA	Dipeptide ABC transporter dipeptide binding	−0.4421 ± 0.09
Genetic Information Processing	VNG0491G	DnaK	Heat shock protein (HSP70 family)	−0.1763 ± 0.06
	VNG0494G	GrpE	Heat shock protein	−0.3835 ± 0.27
	VNG0536G	SirR	Transcription repressor	0.6637 ± 0.03
	VNG0620G	Edp	Proteinase IV homologue	0.2067 ± 0.16
	VNG1690G	Rpl4e	50S ribosomal protein L4	−0.2433 ± 0.05
	VNG1692G	Rpl12p	50S ribosomal protein L2	−0.2203 ± 0.18
	VNG1693G	Rps19p	30S ribosomal protein S19P	−0.2763 ± 0.07
	VNG1695G	Rpl22p	50S ribosomal protein L22	−0.2738 ± 0.18
	VNG1700G	Rps17p	30S ribosomal protein S17	−0.3123 ± 0.17
	VNG1706G	Rps14p	30S ribosomal protein S14P	−0.311 ± 0.03
	VNG1707G	Rps8p	30S ribosomal protein S8	−0.3291 ± 0.18
	VNG1711G	Rpl32e	50S ribosomal protein L32e	−0.2836 ± 0.19
	VNG1713G	Rpl19e	50S ribosomal protein L19e	−0.3415 ± 0.08
	VNG2076G	Rpl40e	50S ribosomal protein L40E	−0.288 ± 0.11
	VNG2096G	CctB	Thermosome subunit Beta	0.1332 ± 0.04
	VNG2226G	CctA	Thermosome subunit Alpha	0.08 ± 0.05
	VNG2473G	RadA1	DNA repair and recombinant protein	0.3357 ± 0.15
Metabolism	VNG0161G	GdhB	Glutamate dehydrogenase	−0.3675 ± 0.06
	VNG0162G	AlkK	Medium chain acyl-CoA ligase	0.2895 ± 0.05
	VNG0474G	PorA	Pyruvate ferredoxin oxidoreductase	−0.3459 ± 0.07
	VNG0559G	Apt	Adenine phosphoribosyltransferase	−0.3551 ± 0.10
	VNG0673G	McmA2	Methylmalonyl-CoA-mutase	0.3732 ± 0.25
	VNG0681G	Hbd1	3-hydroxyacyl-CoA dehydrogenase	0.4570 ± 0.12
	VNG0931G	AcaB2	3-ketoacyl-CoA thiolase	0.3881 ± 0.18
	VNG1557G	CbiH	Cobalamin biosynthesis	−0.3936 ± 0.12
	VNG1567G	CbiC	Precorrin isomerase	−0.8242 ± 0.17
	VNG1644G	NrdB2	Ribonucleotide reductase large chain	0.4806 ± 0.06
	VNG2138G	AtpB	Archeal/vacuolar-type H^+-ATPase subunit B	−0.1414 ± 0.06
	VNG2139G	AtpA	V/A-type ATP synthase	−0.3517 ± 0.14
	VNG2144G	AtpI	H^+-transporting ATP synthase subunit I	−0.1299 ± 0.05
	VNG2203G	PrsA	Phosphoribosylpyrophosphate synthetase	0.6132 ± 0.36
	VNG2372G	Rad24c	DNA repair protein; predicted phosphoesterase	0.1398 ± 0.13
	VNG2471G	NifS	NifS protein, class-V aminotransferase	0.4775 ± 0.38
	VNG2499G	GcdH	Glutaryl-CoA dehydrogenase (GCD)	0.4115 ± 0.04
Unclassified	VNG0527C	VNG0527C	Hypothetical protein	0.3985 ± 0.10
	VNG0815G	YfmJ	Quinone oxidoreductase	0.4365 ± 0.21
Unknown	VNG0153C	VNG0153C	Hypothetical protein	−0.3083 ± 0.29
	VNG0207H	VNG0207H	Hypothetical protein	−0.4162 ± 0.10
	VNG0435H	VNG0435H	Hypothetical protein	−0.4331 ± 0.28
	VNG0597H	VNG0597H	Hypothetical protein	−0.9408 ± 0.21
	VNG1257H	VNG1257H	Hypothetical protein	0.3795 ± 0.32
	VNG1314H	VNG1314H	Hypothetical protein	0.9476 ± 0.23
	VNG1802H	VNG1802H	Hypothetical protein	0.7559 ± 0.13

[a] The log10 of the average ratio of the relative quantification of peptide ions from proteins differentially regulated during growth at 2.6 M NaCl.

The relative quantification of proteins identified with iTRAQ was achieved during analysis by estimating the abundance of the reporter ion peaks (m/z 115,116, and 117). Most of the identified proteins showed a down-regulation in expression in both 2.6 and 5.1 M NaCl (Figure 3). An overall reduction in protein expression was observed following growth in the altered NaCl concentrations. Following incubation at 2.6 M NaCl, 106 of the identified proteins were expressed at lower levels, compared to 62 of the proteins expressed at higher salt. A similar effect was observed following incubation at 5.1 M NaCl (66 proteins higher expressed compared to 75 proteins lower expressed).

General Proteome Changes Associated with Primary Metabolism. A key component of the archaeal and bacterial stress response is the down-regulation of genes that are not necessary for survival, while activating others whose function is to protect the cell.[28,29] In accordance with this premise, we found four flagellin proteins (FlaA2, FlaB1, FlaB2, and FlaB3) down-regulated (Figure 5). Interestingly, neither sodium nor potassium transporters were shown to be differentially expressed at the altered osmotic conditions. Halophilic archaea are known to balance osmotic pressure predominantly by the uptake/release of K^+.[2,30] A likely reason for the results obtained in the present study might be that the cells were incubated for up to 1 week in low-osmotic conditions to reach the desired OD. This may have provided sufficient time for cells to adjust the internal K^+ levels for survival. Another possible scenario would be that an increased intracellular Na^+ concentration might be tolerated during an osmotic stress situation, counterbalancing the lack of K^+. This proposed strategy of *H. salinarum* NRC-1 to tolerate changed external osmotic conditions has been previously described.[16]

Table 2. Proteins That Showed Significant Alteration Following High (5.1 M NaCl) Osmotic Conditions

KEGG classification	gene locus	protein	annotation	log10 ratio[a]
Cellular Processes and Signaling	VNG0960G	FlaB1	Flagellin B1 precursor	−0.1853 ± 0.05
	VNG1009G	FlaA2	Flagellin A2 precursor	−0.1513 ± 0.04
	VNG1467G	Bop	Bacteriorhodopsin	1.2329 ± 0.23
Environmental Information Processing	VNG2063G	Aca	Acetyl-CoA acetyltransferase	0.1557 ± 0.04
	VNG2093G	GlnA	Glutamine synthetase	−0.32 ± 0.05
	VNG2349G	DppA	Dipeptide ABC transporter dipeptide-binding	0.3950 ± 0.36
Genetic Information Processing	VNG0536G	SirR	Transcription repressor	−0.3377 ± 0.14
	VNG0620G	Edp	Proteinase IV homologue	0.2343 ± 0.12
	VNG1105G	Rpl1p	50S ribosomal protein L1	−0.1301 ± 0.06
	VNG1108G	Rpl11p	50S ribosomal protein L11P	−0.1383 ± 0.09
	VNG1138G	Rpl13p	50S ribosomal protein L13P	−0.1286 ± 0.08
	VNG1690G	Rpl4p	50S ribosomal protein L4	−0.1322 ± 0.07
	VNG1693G	Rps19p	30S ribosomal protein S19P	−0.1132 ± 0.04
	VNG1697G	Rps3p	30S ribosomal protein S3	−0.1142 ± 0.04
	VNG1698G	RpmC	50S ribosomal protein L29	−0.1313 ± 0.08
	VNG1700G	Rps17p	30S ribosomal protein S17	−0.1378 ± 0.13
	VNG1703G	Rps4e	30S ribosomal protein S4	−0.1098 ± 0.08
	VNG1714G	Rpl18p	50S ribosomal protein L18	−0.1535 ± 0.06
	VNG1716G	Rpl30p	50S ribosomal protein L30P	−0.1549 ± 0.10
	VNG1997G	InfB	Translation initiation factor IF-2	−0.2621 ± 0.14
	VNG2096G	CctB	Thermosome subunit beta	0.0810 ± 0.03
	VNG2226G	CctA	Thermosome subunit alpha	0.27 ± 0.15
	VNG2473G	RadA1	DNA repair and recombination protein RadA	0.1890 ± 0.04
Metabolism	VNG0559G	Apt	Adenine phosphoribosyltransferase	−0.1406 ± 0.07
	VNG0628G	GdhA1	Glutamate dehydrogenase	0.2455 ± 0.08
	VNG0997G	Acs2	Acetyl-CoA synthetase	0.2676 ± 0.13
	VNG1089G	PurA	Adenylosuccinate synthase	−0.2653 ± 0.12
	VNG1325C	Thyx	Thymidylate synthase	−0.1742 ± 0.08
	VNG1814G	CarB	Carbamoyl-phosphate synthase large subunit	−0.218 ± 0.07
	VNG1815G	CarA	carbamoyl-phosphate synthase small subunit	−0.2117 ± 0.10
	VNG2139G	AtpA	V-type ATP synthase subunit A	0.1086 ± 0.05
	VNG2374Gm	LysC	Asparate kinase	−0.3083 ± 0.09
	VNG2436G	ArgH	Argininosuccinate lyase	−0.1842 ± 0.07
	VNG2437G	ArgG	Argininosuccinate synthetase	−0.243 ± 0.05
	VNG6053G	CydA_1	Cytochrome d oxidase chain I	0.2757 ± 0.04
Unclassified	VNG0401G	Epf2	mRNA 3′-end processing factor homologue	0.1575 ± 0.07
	VNG0540G	Imp	Immunogenic protein	−0.2049 ± 0.19
	VNG0815G	YfmJ	Quinone oxidoreductase	0.2116 ± 0.08
	VNG1667G	Cdc48c	Cell division protein 48 (CDC48)	0.1759 ± 0.07
	VNG2162C	VNG2162C	Hypothetical protein	−0.1532 ± 0.13
Unknown	VNG0153C	VNG0153C	Hypothetical protein	0.2354 ± 0.06
	VNG0207H	VNG0207H	Hypothetical protein	0.222 ± 0.03
	VNG0597H	VNG0597H	Hypothetical protein	0.2029 ± 0.03
	VNG0743H	VNG0743H	Hypothetical protein	0.3163 ± 0.14
	VNG0995H	VNG0995H	Hypothetical protein	0.5595 ± 0.26
	VNG1564H	VNG1564H	Hypothetical protein	0.3293 ± 0.04
	VNG2282C	VNG2282C	Hypothetical protein	0.2603 ± 0.04
	VNG2379H	VNG2379H	Hypothetical protein	−0.2274 ± 0.09
	VNG2508C	VNG2508C	Hypothetical protein	0.1103 ± 0.07
	VNG2679G	Csg	Cell surface glycoprotein	0.317 ± 0.09

[a] The log10 of the average ratio of the relative quantification of peptide ions from proteins differentially regulated during growth at 5.1 M NaCl.

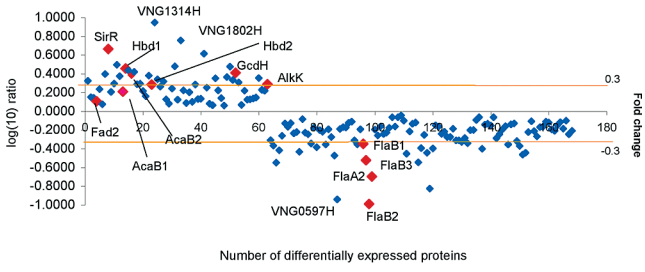

Figure 5. Differential expression of iTRAQ labeled proteins following incubation at 2.6 M NaCl compared to 4.3 M NaCl. Data is given in log(10) scale. The standard deviation for each identified protein is given in the Supporting Information Table 1.

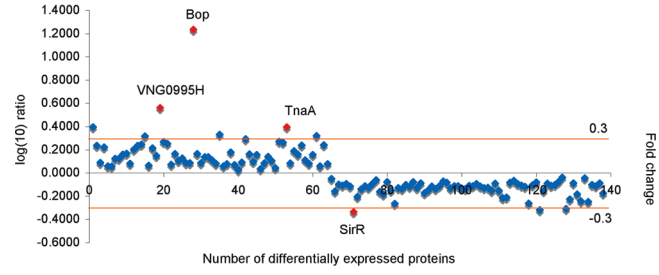

Figure 6. Differential expression of iTRAQ labeled proteins following incubation at 5.1 M NaCl compared to 4.3 M NaCl. Data is given in log(10) scale. The standard deviation for each identified protein is given in the Supporting Information Table 2.

In addition, no changes in the proteins involved in the lipid-modifying pathway were identified during low- or high-osmotic growth. The only known mechanism for the biosynthesis of haloarchaeal polar lipids is via the mevalonate (MVA) pathway.[31,32] It has been shown in previous studies that osmotic shock stimulates the *de novo* synthesis of cardiolipids in halophilic archaea;[33] however, the experimental setup employed for this study (incubation of the samples for up to 10 days) provided enough time for critical cellular functions, such as modified lipid composition, to respond and ensure long-term survival. SirR, a protein that belongs to a family of regulators in transcriptional control of Mn uptake,[34] was up-regulated in the present study during low-osmotic conditions (0.66 ± 0.03) and down-regulated during high-osmotic conditions (-0.33 ± 0.14). It has been shown that SirR down-regulates a Mn-uptake ABC transport system in the presence of Mn(II).[34] It is well-known that several factors such as salinity, temperature, and pH can alter effective metal ion concentration, and that high levels of metals can be toxic to cells.[34] These results indicate that during low salt conditions Mn(II) uptake is repressed to protect the cells from metal ion stress.

Bacteriorhodopsin (Bop) was up-regulated at 5.1 M NaCl (1.23 ± 0.23) (Figure 6) and potentially up-regulated at 2.6 M NaCl (0.31 ± 0.21) (Figure 5). Bacteriorhodopsin converts the energy of light (500−650 nm) into an electrochemical proton gradient, which in turn is used for ATP production by ATP synthase,[35] yet this strong up-regulation was unexpected since bacteriorhodopsin is only induced in this organism when it is grown under anaerobic conditions with light.[36,37] A possible explanation for the observed up-regulation at 5.1 M NaCl is that oxygen solubility strongly depends on the sodium chloride content,[38] therefore, depleting the organism of available oxygen. Furthermore, the infrequent exposure of these cultures to ambient light may have also contributed to the observed result. The potential up-regulation of bacteriorhodopsin following 2.6 M NaCl (0.31 ± 0.21) however remains unclear, as the described conditions do not favor the expression of this protein, and may represent a false-positive result. Although there was a strong up-regulation of Bop, only one corresponding ATP synthase subunit (AtpA) was shown to be moderately up-regulated at 5.1 M NaCl (0.13 ± 0.05). However, as the expression of primary metabolic genes strongly depends on the growth status of the organism, it cannot be conclusively stated that the observed changes were a direct result of the changes in the external sodium chloride concentration. Similar findings have been reported following the analysis of gene-regulation changes in *Halobacterium* sp. NRC-1 during changes in the external sodium chloride concentration and temperature.[16]

Post-translational Modifications, Protein Turnover, and Chaperones. Under stress situations, proteins often lose their function as polypeptides become mis- or unfolded.[39] Maintaining function by preserving protein structure is accomplished by chaperones and proteases, both of which recognize hydrophobic regions that become exposed on unfolded proteins.[40] During the current experiments, the CctA and CctB protein subunits were marginally up-regulated (0.08 ± 0.05 and 0.13 ± 0.04 during low salt and 0.27 ± 0.15 and 0.08 ± 0.06 during high salt, respectively). CctA and CctB are subunits of the thermosome and belong to the group II chaperonins of archaea involved in various cellular functions during stress,[14,41] including membrane stabilization.[42] Edp, a periplasmic serine protease (Clp protease), was also potentially up-regulated after treatment with both high and low level salt (0.20 ± 0.16 at 2.6 M NaCl and 0.23 ± 0.12 at 5.1 M NaCl). Clp proteases are composed of two components, ClpA and ClpP (an Edp homologue) that degrade proteins in the presence of ATP; however, ClpP alone is capable of rapidly degrading short peptides and cleave longer unstructured polypeptides.[43] The up-regulation of these proteases and chaperones reflects the critical nature of correct protein folding under conditions of altered salinity to afford organism survival.

Fatty Acid Oxidation. Seven proteins involved in the bacteria-like fatty acid β-oxidation pathway were shown to be up-regulated following incubation at 2.6 M NaCl: medium chain acyl-CoA ligase (AlkK), two 3-hydroxyacyl-CoA dehydrogenases (Hbd1 and Hbd2), enoyl-CoA hydratase (Fad2), two 3-ketoacyl-CoA thiolases (AcaB1 and AcaB2), and a glutaryl-CoA dehydrogenase (GcdH) (Figure 5). Previous studies of bacteria under similar stress situations showed that the degradation of fatty acids through β-oxidation generated acetyl-CoA to feed the tricarboxylic acid (TCA) cycle, yielding C-compound intermediates and electron/H^+ ion donors for energy production.[44] In addition, *Haloferax* sp. D1227 is able to utilize aromatic compounds as the sole carbon and energy sources for growth.[45] These upper pathway steps strongly resemble those of fatty acid β-oxidation, yet there are no reports of the oxidation of fatty acids by *H. salinarum* NRC-1. The up-regulation of these proteins is an interesting finding; however, the biological meaning of this up-regulation remains to be determined.

Translation Control and DNA Repair. Much of the translational apparatus was down-regulated in both high and low salt cultures (Supporting Information Tables 1 and 2). Growth at 2.6 M NaCl resulted in a −0.2 to −0.3 reduction of proteins associated with translation (30S and 50S ribosomal proteins) and a down-regulation of the elongation factors eEF1 and TU. Incubation at 5.1 M NaCl only resulted in a −0.1 down-regulation of proteins associated with translation as well as a down-regulation of the elongation factors TU and IF2. However, it has been previously shown that these results could be due to an artifact of slow growth and therefore result in reduced translational capacity.[16]

Although most of the translation machinery was down-regulated following growth at 2.6 M NaCl, several proteins involved in nucleotide biosynthesis were shown to be up-regulated, including the ribonucleotide reductase NrdB and the phosphoribosylpyrophosphate synthetase PrsA. This *de novo* synthesis of nucleotides was suggestive of the DNA damage repair process. Similarly, the DNA repair protein RadA was also found to be up-regulated.

Hypothetical Proteins. Thirty-nine hypothetical proteins were differentially expressed at 2.6 M NaCl, while 32 hypothetical proteins showed altered expression at 5.1 M NaCl (Supporting Information Table 3). In line with the previously observed trend, high osmotic conditions (5.1 M NaCl) did not result in any significant (<0.3) down-regulation of hypothetical proteins, while only two proteins were significantly (>0.3) up-regulated. These proteins were VNG0743H (0.31 ± 0.14) and VNG0995H (0.55 ± 0.26). The PCon/PModeller predicted that the three-dimensional structure for VNG0743H matched that of MJ0577 (PDB: 1mjh): an ATP binding domain of a universal stress protein. VNG0995H had a low confidence structural alignment with a eukaryotic TFIIB transcription factor.

Low-osmotic conditions resulted in 5 hypothetical proteins that were significantly (>0.3) up-regulated: VNG0527C (0.39 ± 0.10), VNG1257H (0.37 ± 0.32), VNG1314 (0.94 ± 0.32),

VNG1339C (0.34 ± 0.19), and VNG1802H (0.75 ± 0.13). VNG1314H showed the highest increase and protein modeling revealed its structural similarity to flavodoxin 2 (pdb: 1YOB) from *Azotobacter vinelandi* (Supporting Information Table 3). Flavodoxins are small electron transfer proteins that contain one molecule of noncovalently but tightly bound flavin mononucleotide (FMN) as a redox active component[46] and are required for a variety of cellular functions, including the activation of the ribonucleotide reductase.[46,47] The ribonucleotide reductase of *H. salinarum* NRC-1 was also shown to be highly up-regulated under these low salt conditions. Structural predictions for the hypothetical protein VNG1802H did not result in any match with a known sequence or functional group.

Conclusion

Proteomic analysis of *H. salinarum* NRC-1 following different osmotic conditions with the iTRAQ-LC/MS systems showed a broad range of proteins involved in the response to prolonged osmotic stress. The strongest responses were recorded following exposure to 2.6 M NaCl, with a global down-regulation of the translational apparatus, up-regulation of chaperones and proteases, and changes in the metabolic activity. One of the most intriguing changes in the metabolic activity was the up-regulation of the bacteria-like fatty acid β-oxidation pathway, previously not known to be actively involved in the haloarchaeal energy cycle. Of specific interest was the large number of uncharacterized proteins that responded to changes in the external osmotic status, in particular protein VNG1802H. Further in-depth studies are necessary to elucidate their function and structural adaptation in greater detail. This and other studies[15,16,21] lay the foundation for further investigations into the behavior of halophilic archaea with regards to changes in external salt concentrations. This is of particular interest, as we are now observing halophilic archaea in environments where the NaCl concentrations are far below what was previously considered as optimal.[6,7,48]

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Supporting Information Available: Tables of proteins differentially expressed following incubation at 2.6 and 5.1 M NaCl and hypothetical proteins identified in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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