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Review

Proteomics of Halophilic archaea

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Abstract

Halophilic archaea is a member of the *Halobacteriacea* family, the only family in the *Halobacteriales* order. Most Halophilic archaea require 1.5 M NaCl both to grow and retain the structural integrity of the cells. The proteins of these organisms have thus been adapted to be active and stable in the hypersaline condition. Consequently, the unique properties of these biocatalysts have resulted in several novel applications in industrial processes. Halophilic archaea are also to be useful for bioremediation of hypersaline environment. Proteome data have expended enormously with the significant advance recently achieved in two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS). The whole genome sequencing of *Halobacterium* species NRC-1 was completed and this would also provide tremendous help to analyze the protein mass data from the similar strain *Halobacterium salinarum*. Proteomics coupled with genomic databases now has become a basic tool to understand or identify the function of genes and proteins. In addition, the bioinformatics approach will facilitate to predict the function of novel proteins of Halophilic archaea. This review will discuss current proteome study of Halophilic archaea and introduce the efficient procedures for screening, predicting, and confirming the function of novel halophilic enzymes.

Keywords: Halophilic archaea; Proteomics; Bioinformatics; Prediction of function; Enzymes

Contents

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1. Introduction

1.1. What is halophile?

Halophile (salt-lover) can be defined as microorganisms that require salt to grow. Organisms are able to grow over a wide range of salt concentrations, but with growth rate optima in the absence of salt, are referred to as halotolerant [1]. Microorganisms in this area were defined by Kushner [2] (Table 1).

The term "halobacteria" refers to the red-pigmented extremely Halophilic archaea, members of the *Halobacteriaceae* family, the only family in the *Halobacteriales* order [3]. Most halobacteria require 1.5 M NaCl both to grow and retain the structural integrity of the cells. Halobacteria can be differentiated from halophilic bacteria on the basis of their archaeal characteristics, in particular the presence of ether-linked lipids [4]. Most halobacteria are colored red or orange in cell vesicle due to the presence of C-50 carotenoids, but some strains are colorless, and those with gas vesicles form opaque, white, or pink colonies. A purple hue may be seen in halobacteria that form the bacteriorhodopsin-containing purple membrane [1].

Halobacteria is phylogenetically distinct from bacteria and eukaryotes, and are classified as archaea. They exhibit features characteristic of the archaea, including eukaryotic-like transcription and translation machinery, ether-linked lipids and like some bacteria, a cell wall S-layer composed of glycoprotein [5].

1.1.1. Purple membrane

A unique feature of halobacteria is the purple membrane, specialized regions of the cell membrane that contain a two-dimensional crystalline lattice of a chromoprotein, bacteriorhodopsin. Bacteriorhodopsin which contains a protein moiety (bacterioopsin) and a covalent bound chromophore (retinal) acts as a light-dependent transmembrane proton pump [6]. The membrane potential generated can be used to drive ATP synthesis and support a period of phototrophic growth. Bacteriorhodopsin is induced by low oxygen tension and high light intensity.

Table 1
Categories of Halophilic Microorganisms (Ref. [2])

Category	Salt concentration (M)	
	Range	Optimum
Non-halophile	0–1.0	<0.2
Slight halophile	0.2–2.0	0.2–0.5
Moderate halophile	0.4–3.5	0.5–2.0
Borderline extreme halophile	1.4–4.0	2.0–3.0
Extreme halophile	2.0–5.2	>3.0
Halotolerant	0–>1.0	<0.2
Haloversatile	0–>3.0	0.2–0.5

1.1.2. Carotenoids

Halobacteria produce large quantities of red–orange carotenoids. Carotenoids have been shown to be necessary for stimulating an active photorepair system to repair thymine dimmers resulting from ultraviolet radiation. The most abundant carotenoids are C-50 bacterioruberins, although smaller amounts of biosynthetic intermediates such as beta-carotene and lycopene are also present. Retinal is produced by oxidative cleavage of beta-carotene, a step that requires molecular oxygen. Several retinal proteins, in addition to bacteriorhodopsin, are also produce by halobacteria, including halorhodopsin, which is an inwardly directed light-driven chloride pump, and two sensory rhodopsins, which mediate the phototactic response (swimming towards green light and away from blue and ultraviolet light) [7].

1.1.3. Gas vesicles

Halobacteria produce buoyant gas vesicles, like many aquatic bacteria [8]. Gas vesicles are hollow proteinaceous structures surrounding a gas filled space. The function of gas vesicles is to enable the cells to float to the more oxygenated surface layers because halobacteria are primarily aerobic and live in concentrate brines in which the solubility of molecular oxygen is low (especially at high temperatures). This also increases the availability of light for purple membrane-mediated photophosphorylation.

1.2. Adaptation to the hypersaline environment

At high salt concentrations proteins are in general destabilized due to enhanced hydrophobic interactions [9]. Halophilic proteins have, therefore, evolved specific mechanisms that allow them to be both stable and soluble in the high cytoplasmic KCl concentration. These adaptive mechanisms are reflected in the solvent interactions and three-dimensional structures of halophilic proteins [10].

When a halophilic enzyme is newly synthesized, many hydrophobic amino acids which are supposed to become buried interior of the native enzyme are exposed to high salt concentrations. This could lead to non-specific inter or intramolecular interactions of these side chains of amino acids which would compete with the proper intramolecular burial of them into correct conformation. It seems, therefore, that halophilic proteins have specific structural elements, to fold into proper native form at high salt concentrations.

Since all soluble halophilic enzymes have highly negative surface charge, once folded properly, their flexibility may be achieved by repulsion forces between close charges. The instability caused by the high surface charge should be somehow balanced to avoid unfolding and it is believed that the role of high salt concentrations is to shield this high surface charge.

All halophilic proteins are highly negatively charged with hydrated carboxyl groups which maintain the solubility of the proteins at high salt concentrations. The electrostatic repulsion offsets destabilization from the hydrophobic effect enhanced by salt. In the case of malate dehydrogenase (hMDH) from *Haloarcula marismortui* whose three-dimensional structure have been determined by X-ray crystallography, the requirement for high NaCl or KCl concentrations for the stabilization can be simply explained by specific, but low affinity binding of salt ions to the folded protein. Therefore, molar amounts of salt are necessary to saturate these binding sites [11,12].

1.3. Potential applications in the biotechnology industry

Halophilic microorganisms produce stable enzymes (including many hydrolytic enzymes such as DNAses, lipases, amylases, gelatinises, and proteases) capable of functioning under high concentrations of salt which leads to precipitation or denaturation of most proteins. Most halophilic enzymes are inactivated and denatured at concentrations of NaCl below 1 M [13]. Examples of halophilic enzymes are hydantoinase from halophilic *Pseudomonas* sp. ATCC 55940 to hydrolyze DL-5-substituted hydantoines to *N*-carbamoyl-D-amino acid [14], extremely halophilic β-galactosidase from *Haloferax alicantei* [15], halophilic α-amylase from *Halobacterium salinarum* [16], extracellular serine protease from the extreme halophile *Halobacterium halobium* (ATCC 43214) [17] and DNA topoisomerase from *Methanopyrus kandleri* [18].

Compatible solutes have gained increasing interest as stabilizers for biomolecules (enzymes, DNA, and membranes) and whole cells, salt antagonists, or stress-protective agents. One of the most promising applications is the use of ectoine from *Halomonas elongata* as stabilizers in the polymerase chain reaction (PCR) [19]. Other examples are betaine to improve osmotic tolerance of commercially important crops (such as potato, rice, tomato, and tobacco), diglycerol phosphate as a potentially protein stabilizer [20], trehalose as a cryoprotectant for the freeze-drying of biomolecules [21].

Biosurfactants and halophilic exopolysaccharides enhance the remediation of oil-contaminated soil and water. Biosurfactant-producing halophili/halotolerant microorganisms may thus play a significant role in the accelerated remediation of oil-polluted saline environments. Since many petroleum reservoirs are anaerobic and have high salinity and temperature, the application of biosurfactants for in situ microbially enhanced oil recovery requires organisms that grow and produce these surfactants under these environmental conditions.

Liposomes are used in cosmetics and medicines for the transport of compounds to specific target sites in the body. Ether-linked lipids from archaeal halophiles have a high chemical stability and resistance against esterases and thus a higher survival rate than liposomes based on fatty acid derivatives [21,22]. Novel ether lipids were obtained from the extreme halophile *Halobacterium cutirubrum*.

The ability of halophiles/halotolerants to oxidize hydrocarbons in the presence of salt is useful for the biological treatment of saline ecosystems contaminated with petroleum products. Many cases of successful bioremediation of hydrocarbons have been reported using halophiles in marine, Arctic, and Antarctic environments [23,24], in salt-rich stratum fluids [25], in Lousiana salt marshes [26,27], in a biofilm reactor [28], in saline phenolic wastewater [29].

Halogenated organic compounds are of environmental concern because of their persistency and toxicity. A slightly halophilic and alkaliphilic *Nocardioides* sp. could utilize 2,4-dichlorophenol; 2,4,5-trichlorophenol (from agricultural biocides); 2,4,6-trichlorophenol (a wood preservative) as the sole energy source [30], and halophlic archaea, preferably belonging to the genera *Haloarcula*, *Halobacterium* (e.g., strain CBI, DSM11147), and *Haloferax*, are adapted to high concentrations (up to 1 mM) of halogenated hydrocarbons, such as trichlorophenols, or the insecticides lindane and DDT [31]. Aerobic transformation of formaldehyde, an organic solvent by a moderately halophilic eubacterium in presence of 1–20% (w/v) salt was also reported [32].

Halotolerant microorganisms catalyze various fermentation processes that occur in the presence of salt, thereby producing compounds of characteristic taste, flavor, and aroma. *Lactobacillus plantarum* had an essential role in the fermentation of pickles and Sauercraut. *H. salinarum*, *Halococcus* sp., *Bacillus* sp., pseudomonads, and coryneform bacteria are used in the production of an Asian (Thai) fish sauce (nam pla).

Antarctic marine bacteria, *Shewanella* and *Colwellia* contain significant proportions of long-chain polyunsaturated fatty acids which could be used as dietary supplements to counteract deficiency in essential fatty acids [33]. They have merits over diminishing fish oil source because they allow conventional purification procedures, rapid production rates, and consistency of product quality and yield.

The production of alternative energy is another application field of halophiles. Hydrogen is considered to be a likely

future energy source because it is easily converted to electricity and easily combustible. The use of photosynthetic bacteria has the advantage that H_2 production can be carried out in light using organic substances that are present in various biological resources. A halophilic bacterial community including photosynthetic bacteria was able to utilize raw starch directly for H_2 production in a single-step culture system in the presence of 3% (w/v) NaCl and light [34].

Amelioration of soil salinity during crop growth by application of a N-fixing cyanobacterium, *Anabaena torulosa*, was proposed. Removal of top soil with the cyanobacterial mats (whereby the fixed N was also removed) decreased the soil salinity significantly (26–38%) [35].

One possible strategy to recover saline land for agricultural use is the transfer of halotolerance from halophilic organisms to crops of agronomic value. Transgenic tobacco plants acquired resistance to salt stress after introduction of dnaK1 gene from the halotolerant cyanobacterium *Aphanothece ahlophytica*, which can grow in saline conditions up to 3 M NaCl [36].

2. Proteomics of Halophilic archaea

2.1. Protein expression map

Recently, the whole genome sequencing of *Halobacterium* species NRC-1 has been completed [37]. This would be tremendous help for identifying the proteins of the similar strain *H. salinarum* using mass spectrometry. The *H.* species NRC-1 genome comprises a 2014 kb chromosome and two minichromosomes: pNRC200 and pNRC100 sizing 365 and 191 kb, respectively. It contains approximately 2413 nonredundant predicted protein-coding genes [37]. This was obtained by computational annotation of *H.* species NRC-1 genome using the gene-finding program GLIMMER, which finds ORFs, and executes homology comparisons against pre-existing cDNAs and selected genome sequences [37]. These predicted genes can be categorized into three classes: (i) genes homologous to previously characterized *H.* species NRC-1 and non-*H.* NRC-1 genes of known function and/or containing motifs or domains associated with certain functions (1067 genes); (ii) genes homologous to non-*H.* NRC-1 genes of unknown function (590 genes); and (iii) hypothetical genes with no similarity to pre-existing known genes or domains (971 genes) [38]. Interestingly, calculated isoelectric points of about 2600 predicted genes from *H.* species NRC-1 gave the median pI of 4.9 proving highly acidic nature which is common to all the halophilic proteins [39]. In accordance with the ability of *H.* species NRC-1 to grow on amino acids, which ultimately are catabolized by the citric acid cycle, the genes coding all of the enzymes for an aerobic cycle are present (Fig. 1). In common with all archaea, the conversion of pyruvate to acetyl-CoA (before the citric acid cycle) and of 2-oxoglutarate to succinyl-CoA are catalyzed by the respective 2-oxoacid ferredoxin oxidoreductases [40,41]. Interestingly, genes encoding malate ferredoxin oxidoreductase and fumarate reductase are also present, so that when combined with the 2-oxoglutarate oxidoreductase, they could form a partial reverse citric acid cycle from oxaloacetate to 2-oxoglutarate under anaerobic conditions, as has been found in a number of methanogenic archaea [42,43]. In connection with the citric acid cycle, the key enzymes of the glyoxylate cycle, isocitrate lyase, and malate synthase, could not be identified in the genome sequence. This is in accord with an inability of *Halobacterium* to grow on acetate [44,45].

Among other findings that have been reported about the physiology of the *H.* species NRC-1 a systems approach using cDNA microarray and differential proteomics by ICAT analyses revealed the coordinated coregulation of several interconnected biochemical pathways for phototrophy: isoprenoid synthesis, carotenoid synthesis, and bacteriorhodopsin assembly [38]. Recently, proteome analyses of *H.* species NRC-1 by shotgun approach using LC–MS/MS identified a range of membrane and cytoplasmic proteins related to various metabolic pathways [10]. A total of 401 chromosome proteins and 25 minichromosome pNRC100 and pNRC200 proteins were identified. In order to obtain functional and physiological information regarding these expressed proteins, 295 of the expressed proteins with putative functions were searched against the KEGG Enzymes/Compounds/Genes Pathway Database (http://www.genome.ad.jp/kegg-bin/mk_point_html). A number of metabolic pathways showed more than 50% of their members present in the group of expressed proteins, and thus suggests such pathways were active at the time the cells were collected or identified proteins are constitutively expressed [46]. Proteome analysis data supports the previous observation that pNRC100 and pNRC200 contain indispensable gene sequences [47,37]. Among the approximately 40 putative genes on the pNRC100 and pNRC200 likely to be essential or important for cell viability, 10 proteins were identified in this study. These include the pNRC200 proteins, arginine deiminase (ArcA), ornithine arbamoyltransferase (ArcB), carbamate kinase (ArcC), aspartate carbamoyltransferase catalytic subunit (PyrB), spartate carbamoyltransferase regulatory chain (PyrI), cytochrome *d* oxidase chain I (CydA; also on pNRC100), sn-glycerol-1-phosphate dehydrogenase (GldA), arginine-tRNA synthetase (ArgS), Na_/H_ antiporter (NhaC3), and glycerol-3-phosphate-binding protein (UgpB). There were 29 proteins with tryptic peptides identified from both membrane and soluble protein fractions. These included cell surface glycoprotein (Csg), 15 ribosomal proteins, four flagella proteins, a gas vesicle protein, GvpC, and eight other proteins. Among these proteins, only Csg, FlaA1a, and FlaB1 contained both TMHMM and TMpred membrane domains and DppA, FlaA2, FlaB3, SdhA, and NrdB2 contained Tmpred membrane domains (scores_1000) but no TMHMM predicted membrane domain. It is unusual to find flagellar proteins in the soluble fraction. They may be present in the

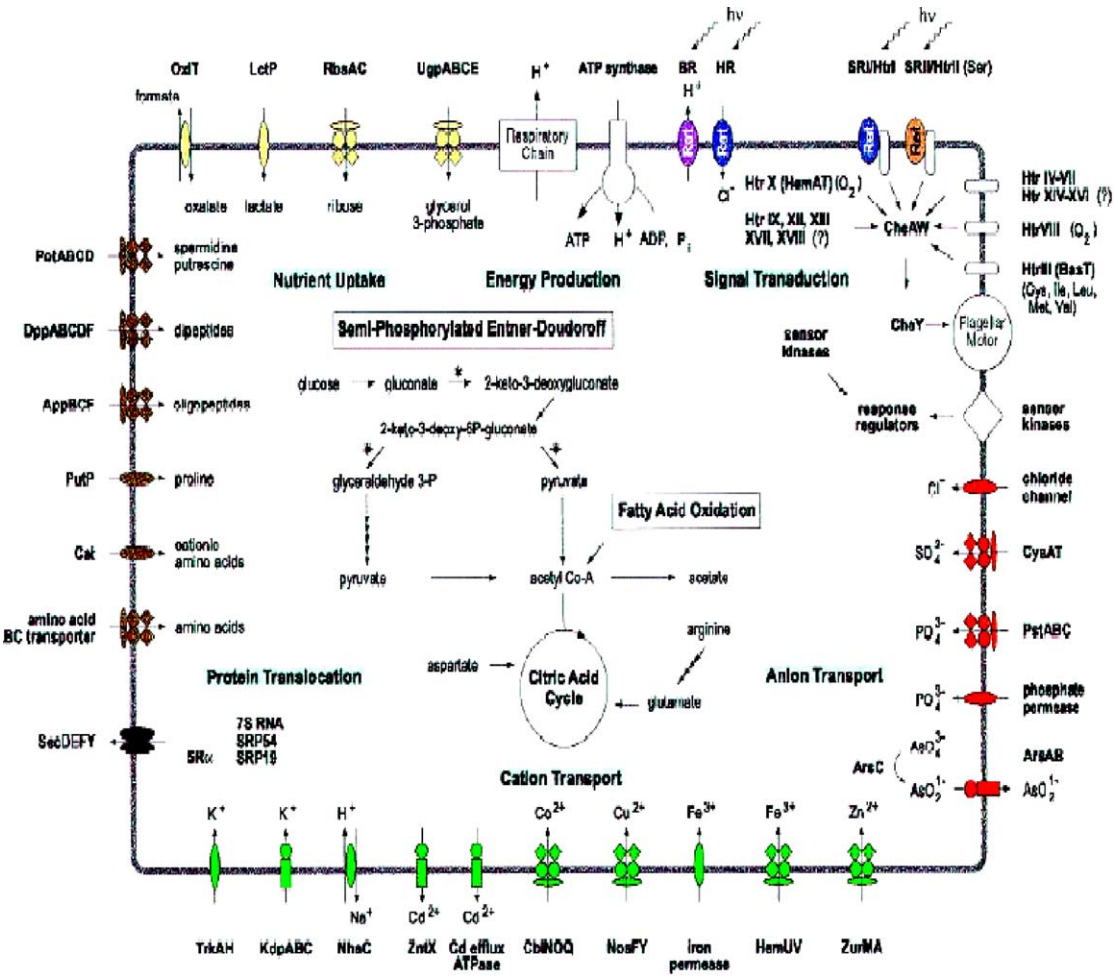

Fig. 1. An integrated view of the biology of *Halobacterium* species NRC-1 (Ref. [37]). Aspects of energy production, nutrient uptake, membrane assembly, cation and anion transport, and signal transduction are depicted. Several amino acids may be used as a source of energy, including arginine and aspartate, which are passed to the citric acid cycle via 2-oxoglutarate and oxaloacetate, respectively.

soluble fraction as flagella fragments that have been detached from the basal body [48] where they are connected to the cytoplasmic membrane, or alternatively as unassembled precursor proteins. In *Halobacterium*, the formation of gas vesicles is induced under low oxygen conditions, enabling cells to float to the surface and grow phototrophically [49,50]. Interestingly, the major gas vesicle protein, GvpA, was identified only in the membrane fraction. The GvpC peptides detected in the soluble protein mixture might have come from GvpC molecules detached from gas vesicle surfaces [49].

The other trial has achieved by low-pass shotgun sequencing in four different type of Halophilic archaea: [51] the metabolically versatile *H. marismortui*; [37] the non-pigmented *Natrialba asiatica*; [52] the psychrophile *Halorubrum lacusprofundi* and [53] the Dead Sea isolate *Halobaculum gomorrense*. Approximately one thousand single pass genomic sequences per genome were obtained. The data were analyzed by comparative genomic analyses using the completed [54].

2.2. Two-dimensional gel electrophoresis analysis of *H. salinarum*

Given the recent advances in genomics and proteomics technologies and active ongoing researches on *H. salinarum*, the whole proteome approach has been initiated to find and develop novel enzymes from this organism [55–58]. Traditional methods for finding novel industrial enzymes focus on activity screening where the specific enzymatic function of choice is applied to various strains of microorganism. On the contrary, the 2-D gel electrophoresis coupled with ESI-Q-TOF MS/MS was performed to obtain global view of the synthesis and distribution of many proteins in the *H. salinarum*.

2.2.1. Cultivation of *H. salinarum*

H. salinarum (ATCC 33171) was grown in a medium containing 1 g/L K_2HPO_4, 0.5 g/L $MgSO_4$, 10 g/L yeast extract and 3.5, 4.3, and 6.0 M of NaCl, pH 7.2. The growth rate of *H. salinarum* was different according to salt concentrations in the culture medium (Fig. 2) [56]. The specific growth rate

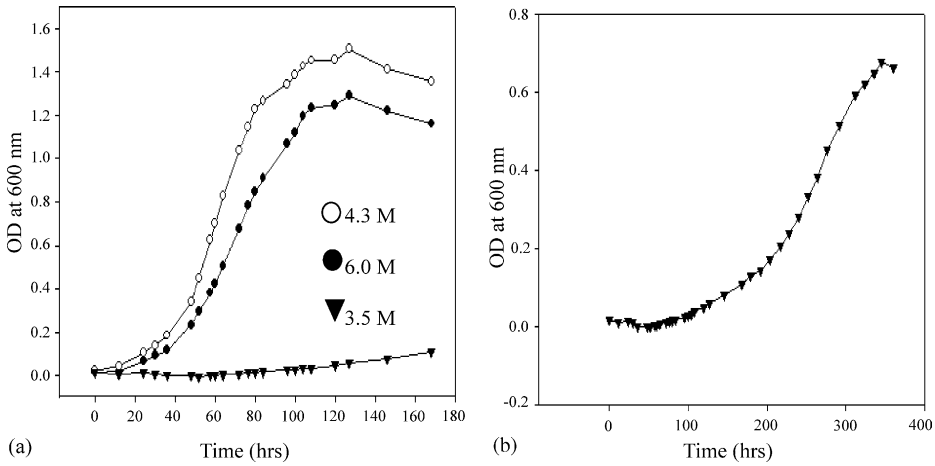

Fig. 2. Growth curve of *H. salianrum* cultured at a salt concentration of 3.5, 4.3, and 6.0 M (a), extended view of 3.5 M condition (b) (Ref. [56]) *H. salinarum* was harvested in the mid-exponential phase at 72 h of cultivation at 4.3 and 6.0 M NaCl, and 240 h at 3.5 M NaCl. From this growth curve, it was confirmed that 4.3 M of NaCl was optimum for growth of *H. salinarum*.

of *H. salinarum* was 0.015 at 3.5 M, 0.046 at 4.3 M, and 0.033 at 6.0 M NaCl. Therefore, it could be confirmed that 4.3 M of NaCl was optimum for growth of *H. salinarum*.

2.2.2. Sample preparation for 2-DE

The cells were incubated in 35 °C with shaking at 180 rpm and harvested in mid-exponential phase by centrifugation at 8000 rpm for 10 min. Proteins were prepared following the procedure reported previously [55]. The pellet was resuspended in 10 volume of lysis buffer containing 10 mM EDTA, 20 mM Tris–Cl (pH 8.0), 0.04% protease inhibitor cocktail (PIC), 0.5 mM DTT, 0.1% CHAPS and sonicated in an ultrasonic disrupter (Branson, Danbury, CT, USA) for 3 min. Cell debris was removed by centrifugation at 13,000 rpm for 20 min at 4 °C and the supernatant was treated with DNase I (100 μg/mL) and RNase A (25 μg/mL) for 1 h on ice. Sample was desalted four times using centrifugal filter device (Cut-off: 3 kDa, Amicon, Millipore Corp., USA), and the proteins were precipitated by adding 10% TCA, 10% cold acetone, and 20 mM DTT at 4 °C. After washing and drying, pellet was re-solubilized in the buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.04% PIC, and 20 mM Tris–HCl (pH 8.0). Insoluble aggregates were removed by centrifugation and this was desalted again two to four times as described above.

2.2.3. IEF and SDS–PAGE

IEF was carried out using the IPGphor IEF System (Amersham Biosciences, Piscataway, USA) using IPG strips pH 3–10 L, 24 cm (80 μg) for the total of 84,000 Vh. For ultrazoom IPG strips of pH 3.5–4.5, 24 cm, active rehydration was conducted on the IPGphor and strips were moved to the Multiphor system (Amersham Biosciences, Piscataway, USA) for isoelectricfocusing for the total of about 120,000 Vh. The second dimensional electrophoresis was performed on the 12.5% SDS–PAGE gels (26 cm × 20 cm) and SDS–PAGE was performed at 55 V for 1 h, 160 V for 1 h, and 380 V for 4 h using Ettan DALT II system (Amersham Biosciences, Piscataway, USA). Proteins were visualized using the silver staining method [59] with some modifications. The comparison analyses of the spots were performed by ImageMaster 2D Elite Software (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.2.4. ESI-Q-TOF MS/MS

Removal of silver ions, in-gel digestion and peptide extraction were carried out as described by Joo et al. with slight modification [59]. Custom-made chromatographic columns were used for desalting and concentration of the peptide mixture prior to mass spectrometric analysis. A column consisting of 100–300 nL of Poros reverse phase R2 material (20–30 μm bead size, PerSeptive Biosystems, USA) was packed in a constricted GELoader tip (Eppendorf, Hamburg, Germany). MS/MS of peptides generated by in-gel digestion was performed by nano-ESI on a Q-TOF2 mass spectrometer (Micromass, Manchester, UK). The source temperature was 80 °C. A potential of 1 kV was applied to the precoated borosilicate nanoelectrospray needles (EconoTip™ New Objective, USA) in the ion source combined with a nitrogen back-pressure of 0–5 pounds per square inch to produce a stable flow rate (10–30 nL/min). Product ions were analyzed using an orthogonal TOF analyzer, fitted with a reflector, a micro-channel plate detector and a time-to-digital converter (Micromass, Manchester, UK). The data were processed using a Mass Lynx Windows NT PC system (Micromass, Manchester, UK) and searched against protein sequences from NCBInr databases using the MASCOT search program (http://www.matrixscience.com).

2.3. Two-dimensional gel electrophoresis profile of *H. salinarum* proteome

The 2-DE profile of *H. salinarum* proteins on the IPG strip ranging pH 3–10 demonstrated a majority of proteins

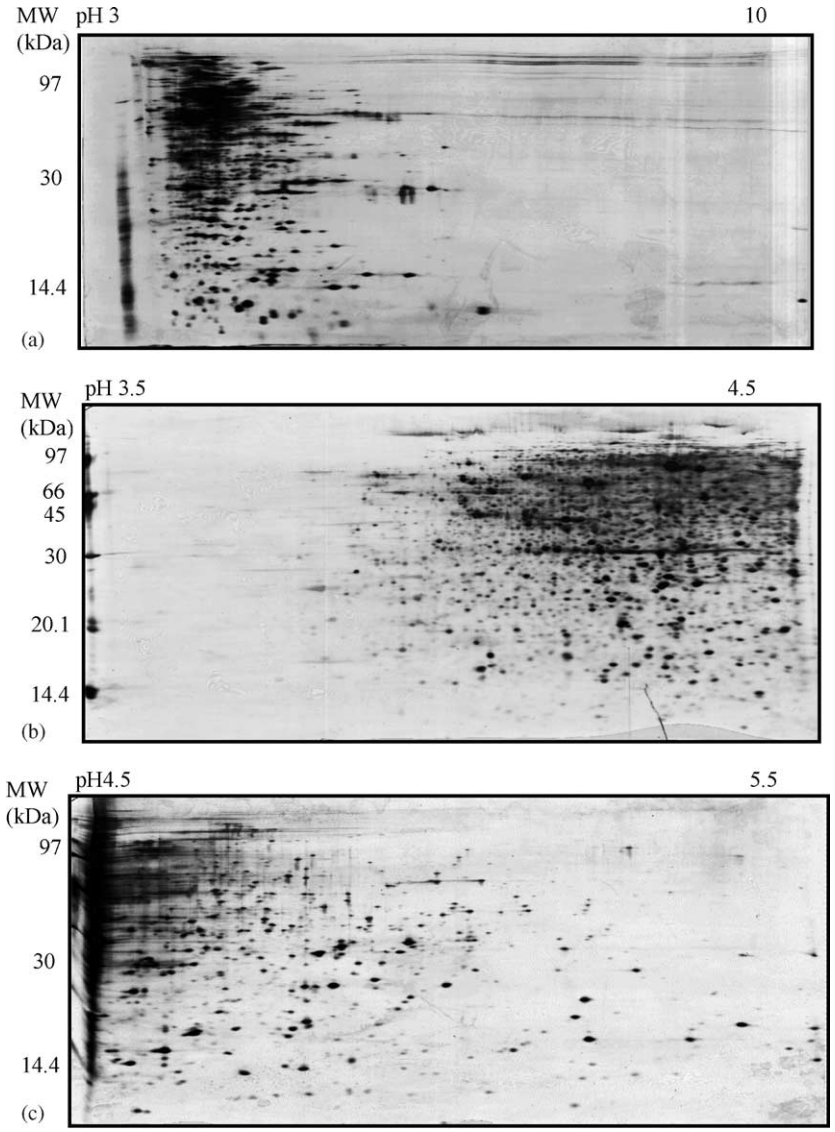

Fig. 3. Protein expression map of *H. salinarum* (Ref. [56]). Eighty microgram of proteins from cell lysate cultured at 4.3 M NaCl was loaded on IPG strip of pH 3–10 (a), 24 cm and separated on MultiPhor. The gel was visualized by silver staining. IEF was carried out for 30 min at 200 V followed by 1 min at 500 V, 1 h to 8000 V and 10 h at 8000 V to a total of 84,000 Vh on pH 3–10 IPG strips using the IPG phor. Most proteins were concentrated in the highly acidic region, around pH 4.2. SDS–PAGE was conducted on 12.5% polyacrylamide gel. (b) IEF using pH 3.5–4.5 ultrazoom IPG strips was carried out with the combined IPG phor/MultiPhor protocol for 148,725 Vh. Ultrazoom drystrip of pH 3.5–4.5 showed better resolution than drystrip of pH 3–10. (c) IEF was performed using pH 4.5–5.5 ultrazoom IPG strips.

distributed between pH 3–6 (Fig. 3a) [56]. These results are in agreement with the report which predicted the characteristics of *H*. species NRC-1 protein based on the genome sequence data [39]. *H*. species NRC-1 was predicted to have an extremely acidic complement of proteins, with a median pI 4.2 using a computational analysis. A reference map of *H. salinarum* proteome was initially constructed and effectively separated with pH 3.5–4.5 and pH 4.5–5.5 ultrazoom IPG strip (Fig. 3b and c). All spots detected in each range were 773 and 464, respectively. As a result of 2-DE analysis, more proteins were detected in pH 3.5–4.5 range than those in pH 4.5–5.5 range. Compare to proteins in pH 3–10 IPG strip, ultrazoom pH 3.5–4.5 and pH 4.5–5.5 IPG strip presented an improved resolution.

2.4. Physiological proteomics

Global regulation of gene expression provides living cells only with those proteins that are necessary for growth or survival. These proteins are produced in a required and tightly adjusted amount and transported to their specific locations inside or outside the cell. Therefore, only a part of genome is active under certain physiological conditions. Proteomics is an excellent method to detect the changes in the protein synthesis pattern of living cells under the influence of different expression conditions. Currently, the application of sensitive matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF)-mass spectrometry and ESI-Q-TOF MS/MS are one of the most important tools for protein identification.

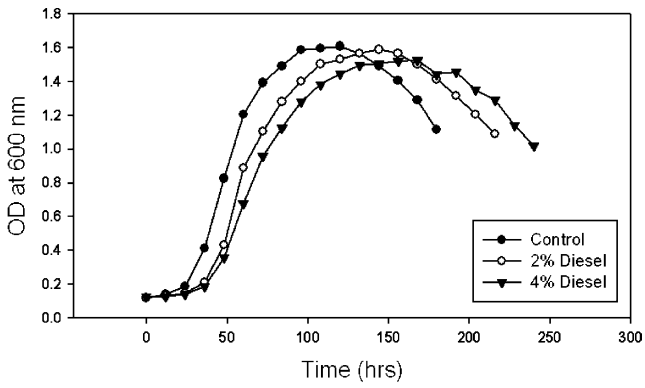

Fig. 4. Growth curve of *H. salinarum* cultured in the media containing diesel (Ref. [58]). *H. salinarum* was cultured in 0% (control), 2 and 4% of diesel. *H. salinarum* survived in all of 0, 2, and 4% diesel media. Cells cultured in 2 and 4% of diesel media were reached to mid-exponential phase after 60 and 72 h, respectively, which were delayed compared to 48 h of control.

Peptide tandem mass spectra were searched against a comprehensive database containing known and conceptual proteins derived from many public databases including the NRC-1 genome sequences.

H. salinarum was cultured in a medium that contains 3.5, 4.3, or 6 M NaCl. The cells were harvested at the mid-exponential phase (10 days at 3.5 M, 72 h at 4.3 M and 72 h at 6.0 M NaCl). With two-dimensional gel electrophoresis over the pH range of 3.5–4.5 and 4.5–5.5, 47 differentially expressed protein spots were identified by ESI-Q-TOF MS/MS analyses (Table 2). Among them ten proteins showed no similarity to pre-existing genes of known function. The identified protein was categorized into 13 parts by their function such as amino acid and nucleotide metabolism [56,57].

A range of organic pollutants has been shown to be mineralized or transformed by microorganisms able to grow in the presence of salt [32]. The usage of microorganisms able to degrade organic wastes containing high salt concentrations could prevent costly dilution to lower the salinity, or the removal of salt by reverse osmosis, ion exchange or electrodialysis before biological treatment. Ward and Brock [60] assumed an inverse relationship between biodegradation of petroleum hydrocarbons and salinity, because enrichment cultures from the Great Salt Lake were not able to grow on mineral oil and to mineralize hexadecane in the presence of salt concentrations above 20% (w/v). The inhibitory effect of salinity at concentrations above 2.4% (w/v) NaCl was found to be greater for the biodegradation of aromatic and polar fractions than of the saturated fraction of petroleum hydrocarbons [61]. Nonetheless, there are several microorganisms reported which are able to oxidize petroleum hydrocarbons even in the presence of 30% (w/v) NaCl. Among such microorganisms are crude oil-degrading *Streptomyces albaxialis* [62] and an *n*-alkane (C_{10}–C_{30})-degrading member of the *Halobacterium* group [63].

As shown in Fig. 4 [58], *H. salinarum* survived in all diesel media, indicating that *H. salinarum* has tolerance to diesel circumstance. Cells cultured in 2 and 4% of diesel media were reached to mid-exponential phase after 60 and 72 h cultivation, respectively, which were delayed compared to 48 h of control. The 2-DE protein profile of cells cultured at 4% diesel was compared with that of control (Fig. 5).

Halobacterium species NRC-1, like other archaea, drives regulated transcription by using a single version of a eukayotic RNA polymerase II-like transcription system. The information for the multisubunit RNA polymerase II is coded by 12 gene including RNA polymerase N [37]. Therefore, RNA

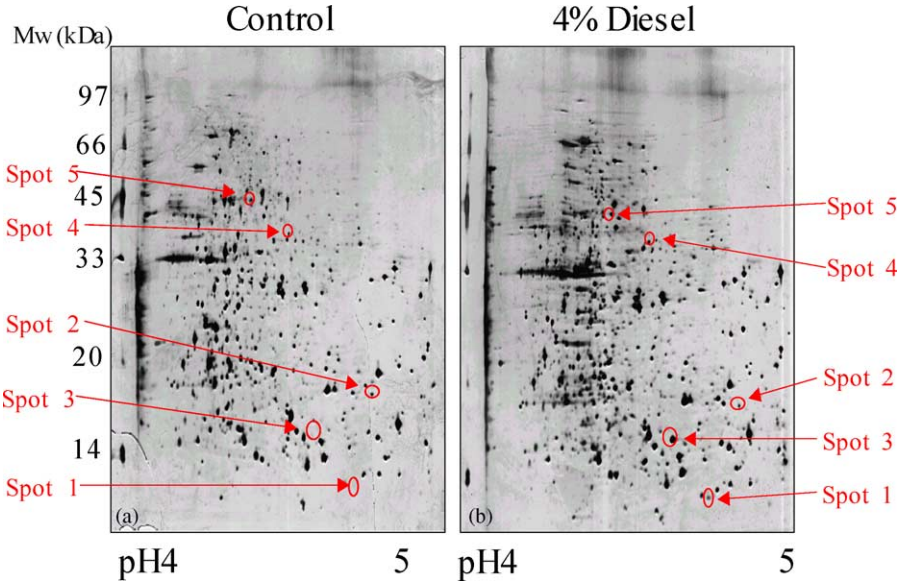

Fig. 5. 2-DE maps of cells cultured in the absence of diesel (a) and in the presence of 4% diesel (b) (Ref. [58]). Five spots were differentially expressed. Spots 1 and 2 were neo-expressed and spots 3–5 were up-regulated about nine-fold, three-fold, and 10-fold, respectively. Protein (80 μg) was loaded on IPG strip (pH 4–5, 24 cm) and SDS–PAGE was performed with a 12.5% gel. The gel was visualized by silver staining.

Table 2
Proteins identified by ESI-Q-TOF MS/MS which show a significant difference in expression with differences in the concentration of salt (Ref. [56,57])

Spot number	Alteration	ID by MS/MS	MW (Da)	pI	Category
427	3.5 M	Ornithine carbamoyltransferase; ArcB	32.82	4.21	
382	(−)	Arginosuccinate synthetase	14.66	4.50	
317		Ornithine carbamoyltransferase; ArcB	32.82	4.21	Amino
421	3.5 M	Ornithine carbamoyltransferase; ArcB	32.82	4.21	acid
194	(+)	Carbamate kinase; ArcC	32.57	4.06	metabolism
445		Ornithine carbamoyltransferase; ArcB	32.66	4.21	
546		Arginine deiminase; ArcA	15.30	4.36	
295	6.0 M (−)	Aldehyde dehydrogenase (retinal)	48.23	4.70	Cell envelop component
409	3.5 M (−)	Thermosome beta subunit	58.81	4.08	Cellular process
325	3.5 M	Thermosome beta subunit	58.75	4.08	
472	(+)	Thermosome subunit alpha; CctA	18.39	4.37	
513	6.0 M (−)	Thermosome beta subunit	58.81	4.08	
347	6.0 M (+)	Thermosome beta subunit	58.81	4.08	
396	3.5 M (+)	Ribonucleoside rrrreductase large chain; NrdB2	22.28	4.07	DNA replication
288	3.5 M (−)	orc/cell division control protein 6	20.40	4.60	
74	6.0 M (−)	Deoxyribonuclease F	41.90	5.20	DNA repair
475	3.5 M (+)	Dihydrolipoamide *S*-acetyltransferase; ArcB	50.46	4.14	
344	3.5 M (−)	Putative oxidoreductase	14.90	4.60	
313	3.5, 6.0 M (−)	Aconitase	23.86	4.70	Energy
465	6.0 M	Dihydrolipoamide *S*-acetyltransferase; ArcB	50.46	4.14	metabolism
541	(−)	Aconitase	20.38	4.23	
579	6.0 M (+)	L-malate dehydrogenase; MdfA	32.23	4.12	
363	3.5 M (−)[a]	Deoxyribose-phosphate aldolase	29.63	4.43	
524	3.5 M (+)[a]	Aspartate carbamoyltransferase catalytic subunit; PyrB	16.33	4.38	Nucleotide
521	6.0 M (−)	Aspartate carbamoyltransferase regulatory chain	21.20	4.21	metabolism
482	6.0 M (+)	Inosine monophosphate dehydrogenase; GuaB	56.02	4.45	
744	3.5 M (−)	Transcription repressor; SirR	25.76	4.24	Regulation
1100	3.5 M (−)	TATA-box binding protein E	24.05	4.28	Transcription
91	3.5, 6.0 M (−)	MRNA 3′-end processing factor homology	50.27	4.60	
441	3.5 M (+)	50S ribosomal protein L5P	19.93	4.32	
351	3.5 M (−)	Ribosomal protein S19	17.61	5.30	
245	3.5, 6.0 M (−)	30S ribosomal protein S7P	18.36	5.00	Translation
366	3.5 M	Aminopeptidase	17.16	5.00	
367	(−)	DNA-binding protein	18.36	5.00	
374	3.5 M (−)	Actin	17.71	4.60	Miscellaneous
200	3.5, 6.0 M (−)	Chymotrypsinogen B	25.49	5.20	
594	3.5 M (−)	Vng2099c[b]	13.58	4.04	Conserved hypothetical protein
520		Vng2296c[b]	17.37	4.17	
441		Vng1093c[b]	25.17	4.35	
567		Vng0932c[b]	18.93	4.35	
1099	6.0 M (−)	Vng1029c[b]	26.00	4.33	
352		Vng0487h[b]	30.98	4.24	
109		Vng1268h[b]	56.34	4.80	
647	6.0 M (+)	Vng2099c[b]	13.58	4.04	
202	3.5, 6.0 M (−)	Vng2536c[b]	23.79	5.00	Hypothetical protein
986	6.0 M	Vng2163h[b]	19.05	4.30	
983	(−)	Vng2163h[b]	19.47	4.30	

[a] Minus in parenthesis means “down-regulated” and plus means “up-regulated” when the expression was compared with that at 4.3 M NaCl. Mowse scores were over 50, which means confidence level of the match from Mascot program.
[b] ORF numbers for the proteins of unknown functions. All the proteins are from *Halobacterium* species NRC-1.

polymerase N (spot 1) is considered to be over-expressed in the diesel media in order to regulate transcription of diesel biodegradation or tolerance genes (Table 3).

Quorum sensing is a cell-to-cell signaling mechanism in which bacteria secrete hormone-like compounds called autoinducers. When these auto inducers reach a certain threshold concentration, they interact with bacterial transcriptional regulators, thereby regulating gene expression. Sperandio et al. [64] observed that genes encoding the expression and assembly of flagella, motility and chemotaxis were also acti-

vated by quorum sensing. Therefore, flagella related protein E (spot 2) may be considered over-expressed in the diesel media because the quorum sensing sensed the presence of diesel as a virulent factors, thus activated the expression of flagella. *H. salinarum* is able to grow anaerobically by fermenting arginine to citrulline using the arginine deiminase pathway. The pathway consists of three enzymes including arginine deiminase [65]. Therefore arginine deiminase (spot 4) is considered to be over-expressed in the diesel media because the diesel oil layer produces an anaerobic status and induces arginine fermentation. Aspartate carbamoyltransferase is related in de novo biosynthesis of pyrimidine ribonucleotides and located in the cell membrane. Aspartate carbamoyltransferase (spot 5) may be excessively released from the membrane into cytosol due to weakening of the membrane with diesel.

3. Bioinformatics in Halophilic archaea

3.1. Current tools of bioinformatics

The rapidly expended field of bioinformatics heralds the advent of innovative breakthroughs in the understanding of microorganism. An up-to date list of Halophilic archea has two websites. One is available on the internet with links to many other websites provided from comprehensive microbial resource (http://www.tigr.org/CMR). Bacterial genomes sequenced at TIGR have been annotated using computer analyses such pair-wise searches and TIGRFAM comparisons in combination with systematic manual evaluation. This administration of analysis has served to generate highly uniform annotation for 14 complete bacterial genomes [66]. These allow not only identification of proteins but further characterization by sorting with enzyme metabolism. Genome information broker (GIB) database is a server that provides a complete dataset of DNA and protein sequences derived from the NRC-1, linked to the relevant annotations and functional assignments, and available at http://www.gib.genes.nig.jp/single/main. It allows one to easily browse through these data and retrieve information, using various criteria such as gene name, keyword, and functional category. It also can be used to search the BLAST protein homologies and to identify the protein functions.

Recently, Fukuchi et al. [67] compared the amino acid compositions of proteins from Halophilic archaea to non-halophilic bacteria including thermophilic eubacteria. In order to compare the amino acid composition in terms of the protein surface and interior, they used the model structures provided by the Genome TO Protein (GTOP) structures and functions database. GTOP is containing an extensive repository of protein folding predictions (sequence

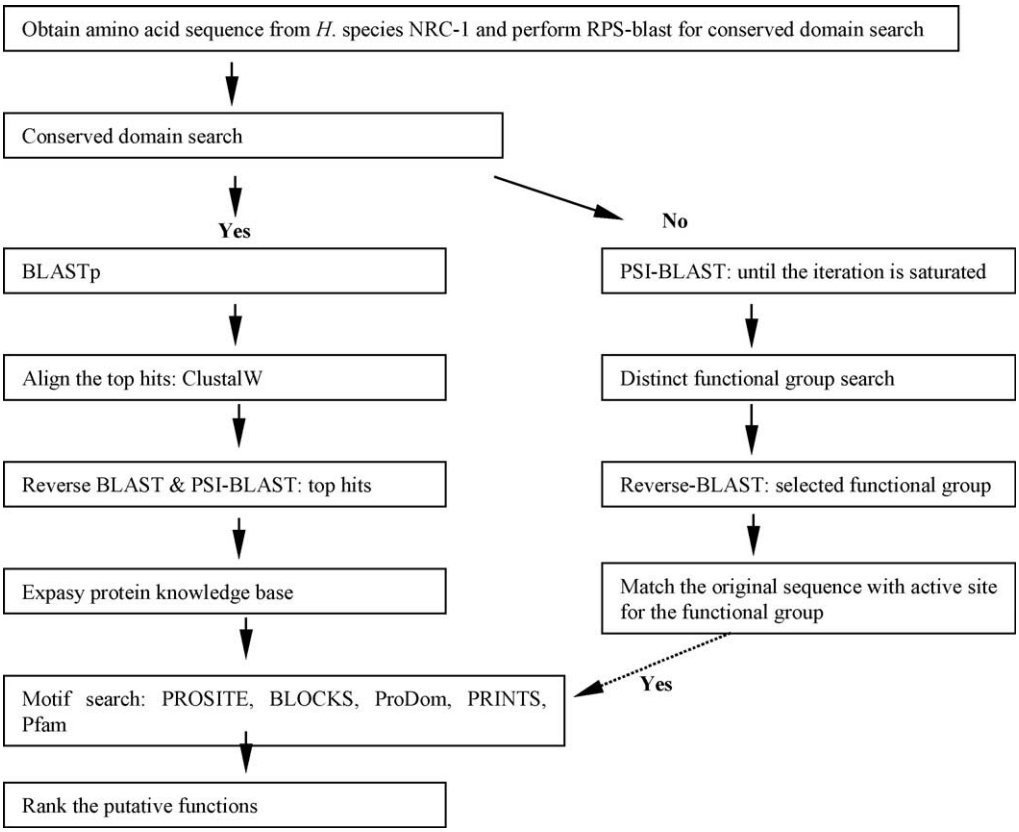

Fig. 6. Flow chart for the prediction of the function of unknown hypothetical proteins (Ref. [57]). Amino acid sequences were attained from *H.* species NRC-1 genome database. The website information for each program is shown in the text.

Table 3
Identification of differentially expressed protein spots of cells cultured in 4% diesel media by MS/MS analysis (Ref. [58])

Spot number	Alteration	MS/MS ID	MW (Da)	pI	Mowse score
1	Neo-expressed	RNA polymerase subunit N	7.41	4.7	60
2		Flagella-related protein E	18.21	4.8	85
3	Up-regulated	Vng1088c	16.21	4.6	85
4		Arginine deiminase	38.08	4.5	296
5		Aspartate carbamoyltransferase	42.20	4.4	291

Spots 3–5 were up-regulated spots about nine-fold, three-fold, and 10-fold, respectively. Mowse score is $-10*\log(p)$, where p is the probability that the observed match is a random event. Individual ion scores >50 indicate identity or extensive homology ($p < 0.05$).

versus structures alignments) obtained chiefly by the program analyses such as homology and motif search, detection of transmembrane helices, coiled-coil region and repetitive sequence, among others. These data, combined with predicted 3D structures, constitute effective tools in characterizing protein functions [68]. They reported that unique protein surface compositions are common in both halophiles and thermophiles. Statistical tests have shown that significant surface compositional differences exist among halophiles, non-halophiles, and thermophiles, while the interior composition within each of the three types of organisms does not significantly differ [67]. Therefore, they concluded that an increase of acidic residues on protein surfaces would not be the only possible way to adapt to high salt-concentrations, and the salt-tolerant proteins mentioned above might have arisen in a particular way, depending on their specific tertiary structures.

3.2. Prediction of novel protein functions from Halophilic archaea

Putative functions of novel proteins screened from *H. salinarum* were predicted using some of many tools currently available on the web (Fig. 6). First, the amino acid sequences of the identified proteins were obtained from the *Halobacterium* species NRC-1 genome and subjected to a BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/) homology search. Many of the novel proteins showed a certain conserved domain at this stage, and those with no functional domain were further analyzed by PSI-BLAST (http://www.ncbi.nih.gov/BLAST/). PSI-BLAST search would give a handful of matching proteins, which can be classified into several groups sharing a certain functional characteristic. This was followed by reverse BLAST, where the representative protein sequences from the functional groups found in PSI-BLAST were queried to see if the resulting matches included the original novel proteins. Resulting candidates showing significant homology were grouped by function and selected in the order of *e*-value, and the protein sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/). The protein sequences were also examined for the existence of conserved motives using the programs: PROSITE (http://www.kr.expasy.org/prosite/), BLOCKS (http://www.blocks.fhcrc.org/), ProDom (http://www.protein.toulouse.inra.fr/prodom.html), PRINTS (http://www.bioinf.man.ac.uk/dbbrowser/PRINTS.html), and Pfam (http://www.genome.wustl.edu/Pfam, http://www.sanger.ac.uk/Pfam/). Finally, the protein knowledge base Expasy (http://www.kr.expasy.org) was consulted for general protein characteristics (Table 3).

As shown in Table 4 [58], predicted functions of the Vng1088 related to diesel degradation primary involved nucleic acid binding and were related to the transcriptional regulation. In addition, 10 novel proteins expressed differently at various salt concentrations can be predicted to have putative function as shown in Table 5. Among them, the protein identified as *H. NRC*-1 "Vng0487h" was suspected to be an acetyl transferase.

Following the procedure illustrated in Fig. 6, three candidate functions were selected for Vng0487h: Hat1, Ydaj, and RimL, in the order of decreasing similarity. Hat1 was found to be a histone acetyltransferase. As demonstrated by its ORF name Vng0486G, it adjoins the upstream end of Vng0487h, implying that these two genes are functionally related. Ydaj from *H.* species NRC-1 was found to be very similar to Hat1 and Hat2, showing the characteristics of a GCN5 histone

Table 4
Summary of predicted functions of Vng1088c (Ref. [58])

Bioinformatic tool	Predicted functions
NCBI PSI-Blast[a]	Unknown
ExPASy Blast[b]	Transcriptional regulator or translation initiation factor
Conserved domain search[c]	COG3388
Conserved domain architecture search[d]	Transcriptional regulator
ScanProsite search[e]	Protein kinase C phosphorylation site
BLOCKS search[f]	Transcriptional coactivator or transcriptional initiation factor
InterProScan search[g]	"Winged helix" DNA-binding domain
DBS-PRED search[h]	DNA binding probability (87.6%)

[a] http://www.ncbi.nih.gov/BLAST/.
[b] http://www.us.expasy.org/tools/blast/.
[c] http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.
[d] http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps.
[e] http://www.us.expasy.org/tools/scanprosite/.
[f] http://www.blocks.fhcrc.org/.
[g] http://www.ebi.ac.uk/InterProScan/.
[h] http://www.gibk26.bse.kyutech.ac.jp/jouhou/jouhoubank.html.

Table 5
Predicted functions of 10 novel proteins

Spot number	Alternation		ID by MS/MS	Putative function
594[a]			Vng2099c	Endoribonuclease
520			Vng2296c	Formyltetrahydrofolate deformylase
441		3.5 M (−)	Vng1093c	Ancient conserved region, cbix containing cobalamin biosynthesisn related domain
567	Salt		Vng0932c	Nucleic acid binding protein containing Zn ribbon related to acetyl-CoA C-acyltransferase
1099	conc[b]	6.0 M (−)	Vng1029c	Basal transcription factor containing helix-turn-helix
352			Vng0487h	Acetyl transferase
109			Vng1268h	Cation/multidrug efflux pump
647[a]		6.0 M (+)	Vng2099c	Endoribonuclease
986		6.0 M (−)	Vng2163h	DNA binding domain, transcriptional regulation
983			Vng2163h	–
3	Diesel[c]	4%	Vng1088c	Transcriptional regulator

[a] Spots 594 and 647 was simultaneously identified as Vng2099c (gi15790939).
[b] (−) in salt conc. means down-regulation in gels of 3.5 or 6.0 M of NaCl condition and (+) means up-regulation [56,57].
[c] Ref. [58].

acetyltransferase. RimL is the name given to the conserved domain, known as the *N*-acetylases of serine residue of the ribosomal protein L7/L12 in *Escherichia coli*. The next step was to confirm the type of acetyl transferase Vng0487h these candidates.

Subsequently, a series of bioinformatic tools were used to predict the function of the novel enzymes and this was confirmed by enzyme activity assay or by protein–protein interaction assay coupled with MS/MS analysis [57]. To identify those proteins capable of interacting with Vng0487h a GST pull-down protein–protein interaction assay was performed in the hope that this would provide the clues concerning the function of Vng0487h.

As a result of GST pull-down assay, a protein was identified to 50S ribosomal subunit protein L3, and 30S ribosomal subunit protein S4 from *E. coli*. Since this was a fusion protein purified from the *E. coli* cell extract, it could possibly have been derived and carried over from the interaction between the fusion protein and *E. coli* proteins. Although the possibility of non-specific binding could not be excluded, it could reasonably be suggested that Vng0487h has a function related to its interaction with ribosomal proteins. From the

Fig. 7. Amino acid sequence alignment of ribosomal protein L12/L7, L3, and L13p (Ref. [57]). Amino acid sequences of ribosomal protein L3 from *E. coli* O 157 (top line), ribosomal protein L13p from *H. salinarum* (middle line), and ribosomal protein L12/7 from *E. coli* K12 (bottom line) were aligned using ClustalW program. Conserved N-terminal serine residues were highlighted by arrows.

result of the function prediction as shown in Fig. 6, RimL, of the three candidates for Vng0487h, is the acetylating enzyme for the N-terminal of ribosomal protein L7/L12. RimL is the protein that acetylates the N-terminal serine of ribosomal protein L12, thereby converting it into L7 [69].

The amino acid sequence alignment of 50S ribosomal protein L7/L12 (RPL7/12) from *E. coli* K12 and 50S ribosomal protein L3 (RPL3) from *E. coli* O157 showed moderate similarity; most serine residues of RPL7/12 at its N-terminal end were conserved as threonines in RPL3. To determine which proteins are homologous to RPL3 or RPL7/12 in the *H. salinarum* genome, these two protein sequences were searched using BLASTp against the whole genome database of *H*. sp. NRC-1. As a result, one protein named ribosomal protein L13p (RPL13p) from *H*. sp. NRC-1 was found to be homologous to RPL3 of *E. coli* with a high confidence level. An InterPro (http://www.ebi.ac.uk/interpro) domain search also showed that RPL13p belongs to the family of ribosomal protein L3; thus reassuring us of the homology of these two proteins. No reliable match was found for RPL7/12 in *H*. sp. NRC-1 (data not shown).

Three proteins, RPL3, RPL7/12, and RPL13p were aligned using ClustalW. As shown in the Fig. 7, the N-terminal serine residue of RPL7/12 was conserved in RPL3 and RPL13p as threonines, which supports the assumption that Vng0487h might acetylate a subunit of ribosomal protein in *H. salinarum*. Taken together, our results confirm the primary function predictions based on sequence homology searches, by an indirect interaction study.

This approach provides an efficient strategy for characterizing the functions of novel proteins highlighted by expression studies using proteomic tools.

4. Conclusions

Halophilic archaea are an interesting class of extremophilic organisms that have adapted to harsh hypersaline conditions. They are able to compete successfully for water and resist the denaturing effects of salts. The diversity of Halophilic archaea in hypersaline environments is also of growing interest. Few hypersaline environments have been carefully surveyed using molecular methods. Finally, halophiles are likely to provide significant opportunities for industry. The proteome analysis of several Halophilic archaea using proteomics tools is in progress. This will provide tremendous opportunities to develop and identify function of novel proteins.

5. Nomenclature

2-DE two-dimensional gel electrophoresis
IEF isoelectric focusing
IPG immobiline dry strip
MALDI-TOF matrix-assisted laser desorption ionization/time of flight
ESI-Q-TOF electron spray ionization/quadropole/time of flight
LC liquid chromatography
MS mass spectrometry
MS/MS mass spectrometry/mass spectrometry
ICAT isotope coded affinity tag
ORF open reading frame

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